

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Atty. Docket: FISHMAN=4

In re Application of:	)	Conf. No.: 4072
	)	
Pnina FISHMAN	)	Art Unit: 1623
	)	
Appln. No.: 09/700,751	)	Examiner: J. Young
	)	
Filed: January 4, 2001	)	Washington, D.C.
	)	
For: PHARMACEUTICAL	)	January 7, 2004
COMPOSITIONS COMPRISING	)	
AN ADENOSINE RECEPTOR ...	)	

DECLARATION UNDER 37 CFR §1.132

Honorable Commissioner for Patents  
U.S. Patent and Trademark Office  
2011 South Clark Place  
Crystal Plaza Two, Lobby, Room 1B03  
Arlington, VA 22202

Sir,

I, Pnina Fishman, an Israeli citizen residing at 19 Asher Barash Street, Herzalia, Israel, hereby declare and state that:

1. I am the inventor of U.S. Patent Application No. 09/700,751 (hereinafter "the application"). I am also the Chief Scientific Officer of Can-Fite BioPharma, the Assignee of the application (hereinafter "Can-Fite").

2. A true and correct listing of my publications is attached hereto as Annex A. My fields of expertise include the field of adenosine receptors and more specifically the A<sub>3</sub> adenosine receptor (A<sub>3</sub>AR) and the development of agonists to this receptor as drugs for the treatment of cancer and inflammatory diseases.

3. The application describes and claims, among others, a method for inhibiting abnormal cell proliferation in a subject, comprising administering to the subject a therapeutically effective amount of an A3-selective adenosine receptor agonist (A3Rag). A specific, but not exclusive embodiment is the use of the A3Rag for treating cancer. Disclosed in the application are a number of examples in which an A3Rag is used for this purpose. However, the effect of A3Rag in inhibiting proliferation of abnormal cells is not limited to the specific embodiments exemplified in the specification.

4. Since filing the application, I have carried out or supervised additional experiments showing the effect of A3Rag in inhibiting proliferation of abnormal cells and thus demonstrating the broad applicability of my invention. In one set of experiments, described in a published manuscript which I co-authored - Fishman, P. et al., Anticancer Research 23, 2077-2083, 2003 (Annex B), androgen-independent PC-3 prostate human carcinoma cells were treated with the synthetic A3Rag, IB-MECA, resulting in inhibition of prostate carcinoma cell growth. The experiments and results reported in Annex B are all true and correct.

5. In another set of experiments, nude Balb/c, Female mice, at the age of 10 weeks were injected subcutaneously with BxPC3 Human Pancreatic Carcinoma tumor ( $3 \times 10^6$  BxPC3 cells/ in 100 $\mu$ L PBS). Tumor size (width [W] and length [L]) was measured every 4 days and was calculated according to the following formula:

$$\text{Tumor Size} = \frac{1}{2} * [(W)^2 * L]$$

Treatment was initiated when tumors reached the size of 150-200 mm<sup>3</sup>. The mice were randomized into 2 groups. One

(control) was treated orally with the vehicle alone while the other was treated orally with IB-MECA (indicated as "CF101", which is the code name for this molecule given to it by the Assignee) at a twice daily dose of 10 $\mu$ g/kg. The growth curves of the control and CF101 treated groups are accurately shown in Annex C. It is clearly evident that pancreatic carcinoma growth was inhibited by IB-MECA.

6. I have also read the Kohno et al. and the Mittelman et al. publications cited by the Examiner in the Office Action dated July 10, 2003.

7. Kohno et al. describe the effect of the A3Rag IB-MECA and Cl-IB-MECA in inducing apoptosis of human promyelocytic leukemia cells. In this article the cells were exposed to concentrations of these agonists in the micromolar ( $\mu$ M) range: IB-MECA at concentrations of 10, 30 and 60  $\mu$ M and Cl-IB-MECA at concentrations of 10 and 30  $\mu$ M (see, for example, Kohno et al., bottom of page 905). These concentrations are several orders of magnitude above the IC<sub>50</sub> of the A<sub>3</sub>AR for these specific agonists, which is in the nanomolar (nM) range as also pointed out by Kohno et al. on page 907, 3 lines from the bottom.

8. Binding studies were carried out for Can-Fite by the French company Cerep, of Le Bois L'Eveque. BP1, Celle l'Evescault, France. The binding study was for a compound designated by Can-Fite as CF101, which is a clinical grade IB-MECA (prepared under clinical good manufacturing practice - cGMP). Their study report (#5367) is attached (Annex D). As can be seen, these binding experiments yielded the following IC<sub>50</sub> and K<sub>i</sub> values:

Receptors	IB-MECA Binding	
	IC <sub>50</sub> (nM)	K <sub>i</sub> (nM)
A <sub>1</sub>	> 1,000	-
A <sub>2A</sub>	685	560
A <sub>2B</sub>	47,600	42,300
A <sub>3</sub>	0.68	0.47

This data clearly shows that IB-MECA has a high binding affinity to the A<sub>3</sub>AR with an IC<sub>50</sub> and K<sub>i</sub> below 1 nM. This data also shows that IB-MECA is highly selective in its binding to the A<sub>3</sub>AR over binding to the other adenosine receptors at low concentration. Based on this data, it is clear that at the concentrations used by Kohno et al., all adenosine receptors, even the A<sub>2B</sub> adenosine receptor, are expected to be activated. Thus at the concentrations used by Kohno, IB-MECA does not exert an A<sub>3</sub>AR mediated specific effect and is not A<sub>3</sub>-selective.

9. Kohno et al. attempt to explain the fact that the concentration required to elicit apoptosis in the HL-60 cells is considerably higher than the IC<sub>50</sub> values. They argue that it *"possibly reflect[s] differences between whole cells and membranes or between species"*. As it can clearly be deduced from the data presented in paragraph 8, above, their conclusion is incorrect. Moreover, this follows also from Kim et al. that I will briefly discuss in paragraph 10 below.

10. Kim S.G. et al., Biochem. Pharmacol. 63, 871-880, 2002 (Annex E), which comes out of the same scientific laboratory as that of the Kohno et al.

publication , further investigated the apoptotic effect of high concentrations ( $\geq 30 \mu\text{M}$ ) of Cl-IB-MECA and concluded that it was not mediated through the A<sub>3</sub> adenosine receptor (see, for example page 877, right column, lines 22-23 - "Therefore, Cl-IB-MECA did not act through a PLC-coupled A<sub>3</sub>AR"; page 878, lines 2-3 - "Therefore, a mechanism other than activation of adenosine receptors seems to be responsible"). Moreover, a number of publications reported that the apoptotic effect of high concentrations (in the  $\mu\text{M}$  range) was seen also in normal cells (see Kim et al., page 877, left column, first 2 lines of the Discussion).

11. It follows from Kim et al. that the apoptotic effect that was seen by Kohno et al., was not mediated through the A<sub>3</sub> adenosine receptor, i.e., it does not exert its prime effect through the A<sub>3</sub> receptor, and the conclusion of Kohno et al. in their article is thus erroneous. It is also clear that the anti-apoptotic effect seen by Kohno et al. at  $\mu\text{M}$  concentrations of IB-MECA and Cl-IB-MECA is not specific for abnormal cells. Furthermore, at the concentrations used by Kohno et al., one would not have expected IB-MECA or Cl-IB-MECA to selectively activate only the A<sub>3</sub>AR, but rather also at least the A<sub>1</sub> and the A<sub>2a</sub> adenosine receptors as well.

12. I attach herewith two manuscripts in which I am the senior author that were recently published: Madi L. et al., J. Biol. Chem., 278, 42121-42130, 2003 (Annex F); and Fishman P. et al., Oncogene, 2003 (published electronically in December, 2003, ahead of print (Annex G). Both of these publications deal with the effect of IB-MECA (identified as "CF101" in the Fishman et al. manuscript) on proliferating cells both in vivo and in

vitro. We have shown in these papers that IB-MECA exerts its anti-proliferative effect *in vitro* at a concentration of 10 nM (see, for example: Madi et al., legends of Figs. 1-8; Fishman et al., legends of Figs. 2-4). Through the use of the specific A<sub>3</sub> adenosine receptor antagonist, we demonstrated, both *in vitro* and *in vivo* that the effect of IB-MECA that we have observed is mediated through the A<sub>3</sub>AR (see Madi et al. Fig. 8 and Fig. 9, *in vitro* and *in vivo* results, respectively; Fishman et al., Figs. 2 and 4, *in vitro* results). The experiments and results in Annexes F and G were all conducted by me or under my supervision and are all true and correct.

13. The specificity of the response was also shown in the Fishman et al. publication of Annex B. As can be seen in Fig. 1 of this publication, the antagonist MRS1523, while almost totally neutralizing the anti-proliferative effect at a concentration of 0.01  $\mu$ M (10 nM), only partially neutralized it at a dose of 1 and 10  $\mu$ M, demonstrating that the effect at high concentrations is at most only partially mediated through the A<sub>3</sub>AR. This corroborates the conclusion of Kim et al. (Annex E) that the effect seen at high concentrations is not receptor mediated.

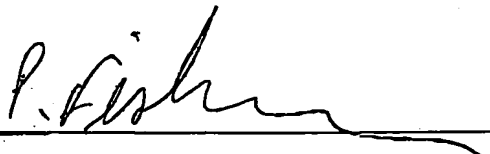
14. In the experiments reported in the Fishman et al. publication of Annex B, in paragraph 5 herein, in the Madi et al. of Annex F and in the Fishman et al. publication of Annex G, anti-proliferative effects of IB-MECA were shown in animals (*in vivo*) at doses of 10  $\mu$ g/Kg body weight, upon oral administration. Similar effects were also seen at doses of up to 100  $\mu$ g/Kg. Based on pharmacokinetic experiments that were carried out on behalf of Can-Fite, these dosages yield plasma levels of

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IB-MECA of about 4 and 40 nM, respectively. These are concentrations which are above the  $K_i$  of the  $A_3AR$  and very much below that of the other adenosine receptors and accordingly concentrations in which IB-MECA will selectively activate the  $A_3AR$  only (namely will not activate the  $A_1$ ,  $A_{2A}$  and  $A_{2B}$  adenosine receptors).

15. Mittelman et al. describe clinical studies that were carried out with  $N^6$ -( $\Delta^2$ -isopentenyl) adenosine (IPA) and  $N^6$ -benzyladenosine in which the anti-cancer effects of these adenosine derivatives was tested in cancer patients. These compounds are not specific  $A_3$  adenosine receptor agonists.

16. The undersigned declares further that all statements made herein of her own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.



Pnina Fishman

Date: Jan. 11, 2003

# **Annex A**

**Prof. Pnina Fishman, Ph.D.**

## **LIST OF PUBLICATIONS**

**December 2003**



## LIST OF PUBLICATIONS

1. Djaldetti, M., Bessler, H., Fishman, P., Lewinski, U and Mandel, M.: Ultrastructural features of the white blood cells in the leukemias. Harefuah, 83:528-537, 1972.
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## **ANNEX B**

## Targeting the A3 Adenosine Receptor for Cancer Therapy: Inhibition of Prostate Carcinoma Cell Growth by A3AR Agonist

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**Abstract.** *Background:* Agonists to A3 adenosine receptor (A3AR) were shown to inhibit the growth of various tumor cell types. The present study demonstrates that a synthetic A3AR agonist, 1-deoxy-1-[6-[[[(3-iodophenyl)methyl]amino]-9H-purine-9-yl]-N-methyl-β-D-ribofura-nuronamide (IB-MECA), inhibits the growth of androgen-independent PC-3 prostate human carcinoma cells and illustrates the molecular mechanism involved. *Materials and Methods:* PC-3 prostate carcinoma cells were used. Cell growth was examined *in vitro* by the thymidine incorporation assay and *in vivo* by inoculating the tumor cells subcutaneously into nude mice and monitoring tumor size. The protein expression level in cells and tumor extracts was tested by Western blot analysis. *Results:* A decrease in the protein expression level of A3AR and the downstream effector PKA $\epsilon$  was observed. Consequently, the GSK-3 $\beta$  protein level increased, resulting in the destabilization of  $\beta$ -catenin and the subsequent suppression of cyclin D1 and c-myc expression. IB-MECA treatment also induced down-modulation of the expression of NF-kB/p65, known to regulate the transcription of cyclin D1 and c-Myc. This chain of events occurred both *in vitro* and *in vivo* and suggests the use of the above-mentioned signaling proteins as markers to predict tumor cell response to A3AR activation. *Conclusion:* Taken together, we demonstrated that A3AR activation de-regulates the Wnt and the NF-kB signaling pathways resulting in the inhibition of prostate carcinoma cell growth.

Activation of the Gi-protein-coupled A3AR has been involved in the inhibition of tumor cell growth (1-3). A3AR is highly expressed in tumor cells whereas low expression has been noted

in a variety of normal cells (4-6). We recently examined the relationship between receptor fate upon activation and receptor functionality in melanoma cells. A3AR activation, with the synthetic agonist IB-MECA, induced rapid receptor internalization to the cytosol. The receptor was then degraded, subsequently re-synthesized and recycled to the cell surface to serve again as a functional receptor. These events generated the modulation of key proteins involved in the Wnt and the NF-kB signal transduction pathways. A decrease in cAMP production and expression of the downstream effector protein kinase A (PKA) and protein kinase B (PKB/Akt) was observed (7-9). We found that when PKA and PKB/Akt were inhibited, GSK-3 $\beta$  level was up-regulated. This led to the phosphorylation and ubiquitination of  $\beta$ -catenin and a decrease in the expression level of cyclin D1 and c-myc, resulting in melanoma cell growth inhibition (3). Moreover, a decline in the expression level of NF-kB was also noted consequent to PKB/Akt down regulation (9). These results were confirmed in an experimental murine model in which IB-MECA inhibited the growth of B16-F10 melanoma metastatic foci in the lung and the development of subcutaneous primary tumor (9). Interestingly, in tumor lesions derived from IB-MECA treated mice, A3AR expression and the level of key signaling proteins (GSK-3 $\beta$ ,  $\beta$ -catenin, NF-kB, cyclin-D1 and c-Myc) were modulated in a pattern corresponding to that observed *in vitro*. These studies demonstrated that there is a direct correlation between A3AR activation, modulation of the signaling proteins and the inhibition of tumor cell growth. We therefore defined 5 of these proteins (PKA, GSK-3 $\beta$ , NF-kB cyclin-D1 and c-Myc) as protein markers to predict the response of tumor cells to A3AR activation both *in vitro* and *in vivo* (9).

Prostate cancer is a common disease in Western countries (10,11) and it is highly resistant to chemotherapy. There is still no effective cure for patients with advanced prostate cancer especially in cases of hormone-independent tumors (12). The molecular mechanisms involved in the initiation, progression and development of prostate cancer are largely unknown. Recently, the Wnt and the NF-kB signaling pathways have also

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**Key Words:** A3 adenosine receptor, IB-MECA, prostate carcinoma, GSK-3 $\beta$ , PKA, NF- $\kappa$ B.

been implicated in the development of prostate carcinoma (13,14). It thus led us to study the effect of IB-MECA on the growth of the human androgen-independent PC-3 prostatic carcinoma cell line and to follow-up the modulation of the 5 protein markers defined above, both *in vitro* and *in vivo*

## Materials and Methods

**Reagents.** IB-MECA and MRS 1523 were purchased from RBI/Sigma (Natick, MA, USA). For both reagents, a stock solution of 10mM was prepared in DMSO and further dilutions in RPMI medium were performed. RPMI, fetal bovine serum (FBS) and antibiotics for cell cultures were obtained from Beit Haemek, Haifa, Israel. Rabbit polyclonal antibodies against murine and human PKAc, c-myc and GSK-3 $\beta$  were purchased from Santa Cruz Biotechnology Inc., Ca, USA. The human and murine rabbit polyclonal antibodies against murine and human cyclin D1 and Rel-65 NF- $\kappa$ B were purchased from Chemicon, Ca, USA. Rabbit polyclonal antibodies against murine and human A3AR were purchased from Alpha Diagnostics, San Antonio, USA.

**Tumor cells and proliferation assay.** PC-3 cells derived from a human androgen-independent prostate cancer cell line (American Type Culture Collection, Manassas, Virginia, USA) were grown in RPMI 1640 penicillin, streptomycin, 2 mM. L-glutamine and 10% fetal bovine serum (FBS). The cells were maintained in T-75 flasks at 37°C in a 5% CO<sub>2</sub> incubator and transferred to a freshly prepared medium twice weekly. For all studies serum-starved cells were used. FBS was omitted from the cultures for 18 hours and the experiment was carried out on monolayers of cells in RPMI medium supplemented with 1% FBS in a 37°C, 5% CO<sub>2</sub> incubator.

[<sup>3</sup>H]-thymidine incorporation assay was used to evaluate cell growth. PC-3 cells (1.5x10<sup>4</sup>/ml) were incubated with IB-MECA (0.01 $\mu$ M-10 $\mu$ M) in 96-well microtiter plates for 24 hours. To test whether IB-MECA exerted its effect on tumor cells through binding to A3AR, an antagonist to A3AR, MRS-1523 (0.1 $\mu$ M), was added to the cell cultures in the presence of IB-MECA. Cultures of PC-3 cells that were incubated in the presence of MRS-1523 only served as controls. For the last 18 hours of incubation, each well was pulsed with 1 $\mu$ Ci [<sup>3</sup>H]-thymidine. The cells were harvested and the [<sup>3</sup>H]-thymidine uptake was determined in an LKB liquid scintillation counter (LKB, Piscataway, NJ, USA). These experiments were repeated at least 10 times.

**Western blot analysis.** To detect the level of expression of A3AR, PKA, GSK-3 $\beta$ ,  $\beta$ -catenin, c-myc and cyclin D1, protein extract from IB-MECA treated or untreated serum-starved PC-3 cells were utilized. The cells were incubated in the presence and absence of IB-MECA for 15 minutes at 37°C. At the end of the incubation period, the cells were then rinsed with ice-cold PBS and transferred to ice-cold lysis buffer (TNN buffer, 50mM Tris buffer pH=7.5, 150mM NaCl, NP 40 0.5% for 20 minutes). Cell debris were removed by centrifugation for 10 minutes, at 7500xg. The supernatant was utilized for Western blot analysis. Protein concentrations were determined using the Bio-Rad protein assay dye reagent. Equal amounts of the sample (50 $\mu$ g) were separated by SDS-PAGE, using 12% polyacrylamide gels. The resolved proteins were then electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). The membranes were blocked with 1% bovine serum albumin and incubated with the desired primary antibody (dilution 1:1000)

for 24 hours at 4°C. The blots were then washed and incubated with a secondary antibody for 1 hour at room temperature. Bands were recorded using BCIP/NBT color development kit (Promega, Madison, WI, USA). The densitometry of protein expression was normalized against  $\beta$ -actin and expressed as % of control (0-time).

**In vivo studies.** The mice were maintained on a standardized pelleted diet and supplied with tap water. Experiments were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at Can-Fite BioPharma, Petah Tikva, Israel.

Nude male Balb/C mice, aged 2 months, weighing an average of 25g were obtained from Harlan Laboratories, Jerusalem, Israel. PC-3 prostate carcinoma cells (2.5x10<sup>6</sup>) were subcutaneously injected into the flank of the mice. When the tumor reached 150-200mm<sup>3</sup> in size, the animals were randomly assigned into different experimental groups. Two types of experiments were set up:

A. a study in which the effect of IB-MECA on tumor growth was evaluated in mice in which the tumor reached a size of 150-200mm<sup>3</sup>. Treatment was given orally once daily for 26 days. This experiment included two groups:

1. Vehicle
2. IB-MECA (10 $\mu$ g/kg body weight).

Tumor size (width (W) and length (L)) was measured twice weekly with a caliber and calculated according to the following formula: Tumor Size = (W)<sup>2</sup>xL/2. Each group contained 10 mice.

B. a study in which the effect of IB-MECA on the expression of tumor markers was evaluated shortly after one treatment in tumor-bearing mice. This experiment included three groups:

1. Vehicle-control.
2. IB-MECA (10 $\mu$ g/kg body weight) given once. Mice were sacrificed after 2 hours.
3. IB-MECA (10 $\mu$ g/kg body weight) given once. Mice were sacrificed after 24 hours.

At the end of each experiment the mice were sacrificed and tumors were excised, protein extracts were prepared as described above and analyzed for the expression profile of A3AR and the marker proteins (PKA, NF- $\kappa$ B, GSK-3 $\beta$ ,  $\beta$ -catenin and cyclin D1).

**Statistical analysis.** The results were evaluated using the Student's *t*-test, with statistical significance at *p*<0.05. Comparison between the mean value of different experiments was carried out.

## Results

**IB-MECA inhibits PC-3 growth *in vitro* and *in vivo*.** To evaluate the direct anti-proliferative effect of IB-MECA on the human androgen-independent PC-3 prostatic carcinoma cell line *in vitro*, we used the thymidine incorporation assay. IB-MECA exerted a dose-dependent inhibitory effect on the prostate carcinoma cells. The inhibition of cell growth was statistically significant at all concentrations tested (*p*<0.001). The A3AR antagonist MRS1523 reversed the inhibitory effect of IB-MECA, demonstrating that tumor growth suppression was specifically mediated through A3AR (Figure 1a).

*In vivo*, the treatment with IB-MECA started when the subcutaneously transplanted PC-3 tumors had grown to a volume of 150-200 mm<sup>3</sup>. As shown in Figure 2a and b, IB-MECA sup-

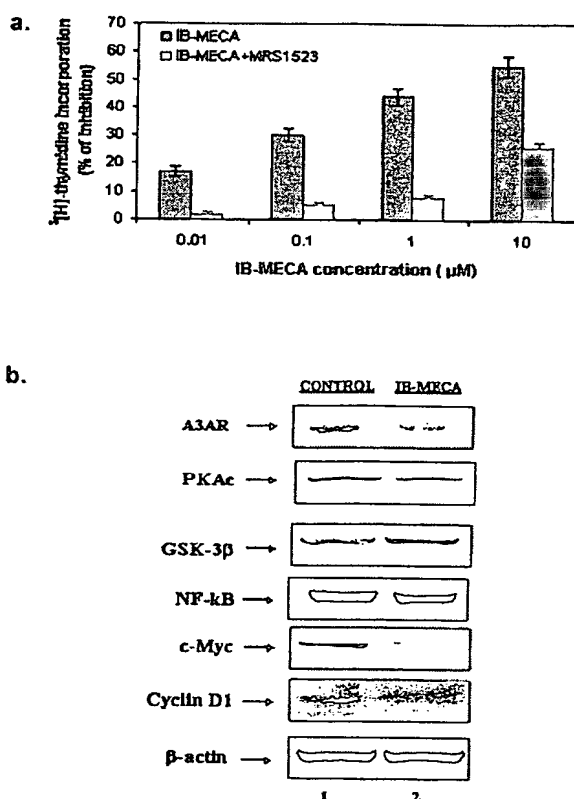


Figure 1. (a) IB-MECA induces a dose-dependent inhibitory effect on the proliferation of PC-3 prostate carcinoma cells. PC-3 prostate carcinoma cells were depleted from serum for 18 hours and treated with vehicle (control) or with various IB-MECA concentrations (0.01 μM-10 μM) in the presence of 1% FBS for 24 hours. Cell proliferation was measured by [<sup>3</sup>H]-thymidine incorporation assay. A3AR antagonist MRS-1523 (0.1 μM) neutralized the inhibitory effect of IB-MECA. The data points are mean ± SEM values from four independent experiments. (b) Expression level of A3AR and protein markers in PC-3 cells upon exposure to IB-MECA. Immunoblots showing the effect of 0.01 μM IB-MECA on the expression level of A3AR, PKAc, GSK-3β, NF-kB, c-Myc and cyclin D1 in PC-3 prostate carcinoma cells. Serum starved cells (for 18 hours) were treated for 15 minutes with IB-MECA in the presence of 1% FBS.

pressed growth of PC-3 tumors during the 26 days of treatment. At the end of the experiment, the mean volume of PC-3 tumors treated with IB-MECA was 69 ± 37 mm<sup>3</sup>, being significantly smaller than that in control group which measured 340 ± 59 mm<sup>3</sup>, the inhibition of tumor growth corresponding to 79.7% ( $p < 0.0001$ , Figure 2a).

**IB-MECA modulates tumor marker proteins upon A3AR activation.** Shortly after A3AR activation with IB-MECA *in vitro*, the expression level of the receptor protein was down-regu-

lated. Additional marker proteins, downstream to A3AR activation, were modulated, *i.e.*, PKA, NF-kB, c-Myc and cyclin D1 expression levels were decreased whereas GSK-3β level was up-regulated (Figure 1b).

In tumor lesions excised from mice treated daily for 26 days with IB-MECA, Western blot analysis revealed down-regulation of A3AR, PKAc, cyclin D1 and c-myc and up-regulation of GSK-3β expression level (Figure 2c). The level of the house-keeping protein β-actin did not change.

To explore the response of the above mentioned tumor proteins to one treatment of IB-MECA, mice with an already established tumor were treated only once with IB-MECA. Two hours after treatment, a marked down-regulation of A3AR, PKA, β-catenin, NF-kB, c-Myc and cyclin D1 was noted. Interestingly, 24 hours after IB-MECA administration, A3AR protein expression level was fully recovered to the control level, whereas the expression level of the other proteins was only partially recovered, and was lower than the control group.

## Discussion

The present study describes the ability of IB-MECA, a synthetic A3AR agonist, to inhibit the growth of prostate carcinoma cells *in vitro* and *in vivo*. A3AR belongs to the family of the Gi-protein-associated cell surface receptors. Receptor activation leads to internalization and the subsequent inhibition of adenylyl cyclase activity, cAMP formation and protein kinase Ac (PKAc) expression (15, 16). IB-MECA is a potent, stable and specific A3AR agonist due to a substitution at the N6 and 5' positions of adenosine. This structure protects the molecule against rapid metabolism by adenosine deaminase and further enhances its affinity to A3AR (17). A3AR expression level was found to be low in most body tissue, whereas tumor cells such as melanoma, T cell lymphoma and pineal tumor cells, significantly express A3AR (4-6). Receptor exhibition and spread is not the only factor determining cell response to a specific ligand. An additional parameter is the exhibition of A2A and A2B adenosine cell surface receptors, known to elicit opposite effects to that of A3AR. At high concentrations, A3AR agonists may also activate A2A and A2B adenosine receptors, affecting the balance of the response (18, 19).

In the present study, the dose-dependent growth inhibition observed in the PC-3 cells *in vitro* was obtained at low concentrations and was counteracted by the antagonist MRS1523. *In vivo*, IB-MECA generated the suppressive effect on tumor growth also at a low-dose (10 μg/kg body weight). It is assumed that since IB-MECA possesses high affinity to A3AR (0.4 nM), it activates this receptor exclusively at low concentrations.

Shortly upon IB-MECA activation, down-regulation of A3AR protein expression level was noted *in vitro*. This observation was confirmed in the *in vivo* studies in which we treat-

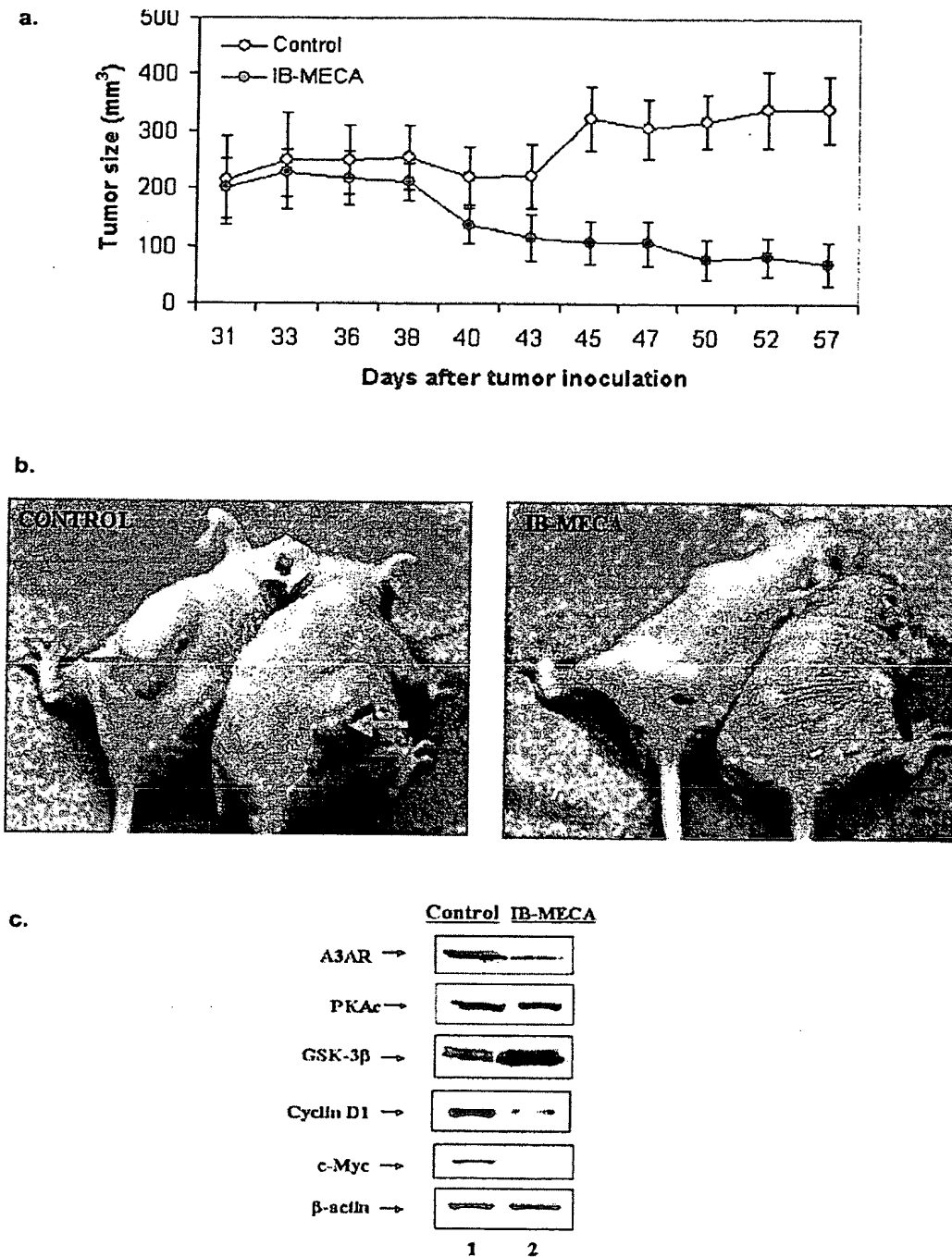


Figure 2. (a) Inhibition of prostate carcinoma cell growth in mice and modulation of tumor protein markers in tumor lesions. (a) PC-3 prostate carcinoma cells ( $2.5 \times 10^6$ ) were subcutaneously injected into the flank of nude mice. One group was treated with IB-MECA ( $10 \mu\text{g/kg}$  body weight) daily orally, starting when the tumor reached a size of  $150\text{--}200 \text{ mm}^3$  and the other, treated with vehicle only, served as control. (b) Representative mice from the control (left) and the IB-MECA (right) treated mice, showing the difference in tumor size in the two groups. (c) Immunoblots showing the effect of IB-MECA on the level of A3AR, PKAc, GSK-3 $\beta$ , cyclin D1 and c-Myc in protein extracts derived from tumor lesions of prostate carcinoma bearing mice (description of the experiment is detailed in a).

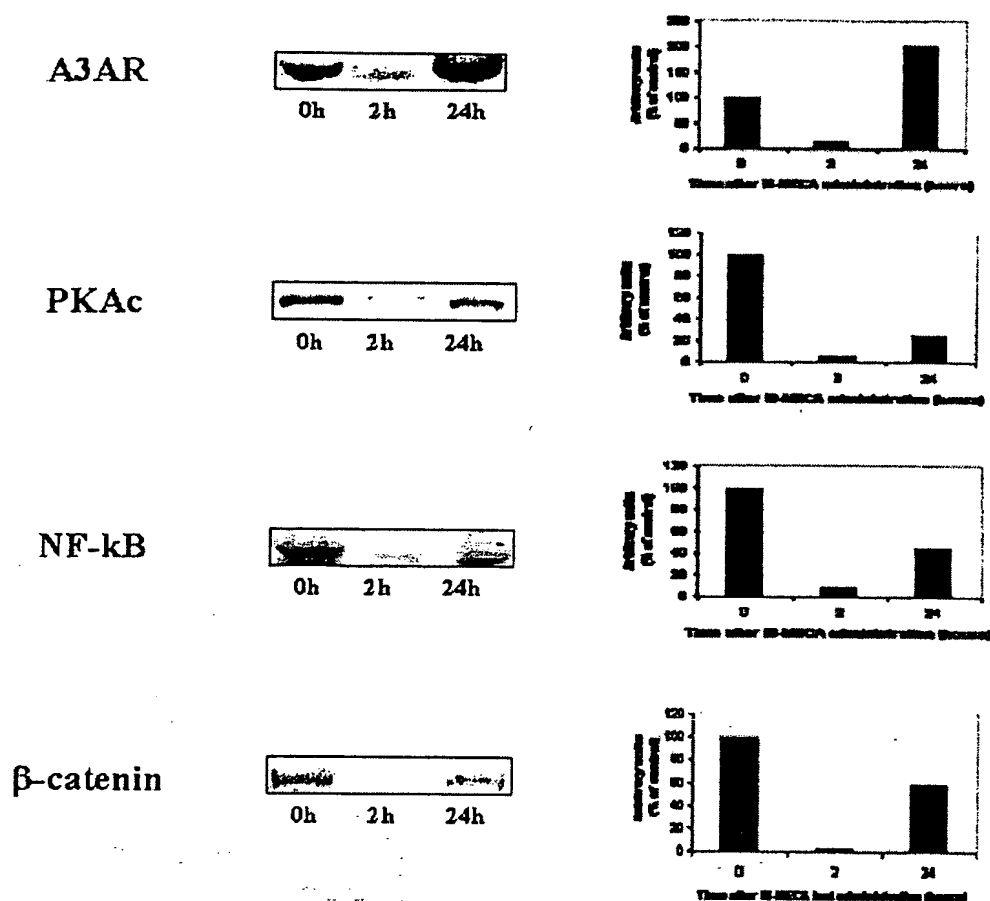


Figure 3. Modulation of tumor protein markers in tumor lesions derived from IB-MECA-treated mice. The effect of IB-MECA (one treatment only, for 2 hours and 24 hours) on the expression of tumor protein markers was evaluated in tumor lesions excised from prostate carcinoma-bearing mice. Immunoblots showing the effect of IB-MECA on A3AR, PKAc, NF-kB, β-catenin, cyclin D1 are presented.

ed tumor-bearing mice with IB-MECA. Receptor down-regulation is a general mechanism typical of Gi protein receptors. This family of receptors responds to ligand activation by receptor internalization (to the cytosol), degradation, re-synthesis and recycling to the cell surface (20). During these events, receptor desensitization/re-sensitization takes place and different signaling pathways are initiated (21, 22). We may suggest that the down-regulation of receptor expression in this study represents the rapid response of the prostate cells to agonist stimulation and the initiation of downstream responses.

Indeed, the prostate cells responded to A3AR activation by a decrease in PKAc level both *in vitro* and *in vivo*. PKAc is an effector protein involved in the initiation/regulation and cross talk between various signaling pathways. It phosphorylates and inactivates the enzyme GSK-3β (23), a key element in the Wnt signaling pathway (24). GSK-3β suppresses mammalian cell

proliferation and survival by phosphorylating the cytoplasmic protein β-catenin, leading to its ubiquitination. GSK-3β in its inactive form does not phosphorylate β-catenin. The latter accumulates in the cytoplasm and subsequently translocates to the nucleus where it associates with Lef/Tcf to induce cyclin-D1 and c-myc transcription (25). In the present study we found that up-regulation of GSK-3β correlated with down-regulation of β-catenin, cyclin D1 and c-Myc. Davies *et al.* reported that there were no mutations within the binding regions between β-catenin and GSK-3β in PC-3 prostate carcinoma cells (26). Therefore, we concluded that there is an involvement of the Wnt pathway in the response of these cells to A3AR activation.

The expression level of NF-kB was down-regulated in both *in vitro* and *in vivo* studies. NF-kB is also linked to the effector protein PKAc. The most abundant form of NF-kB is a het-

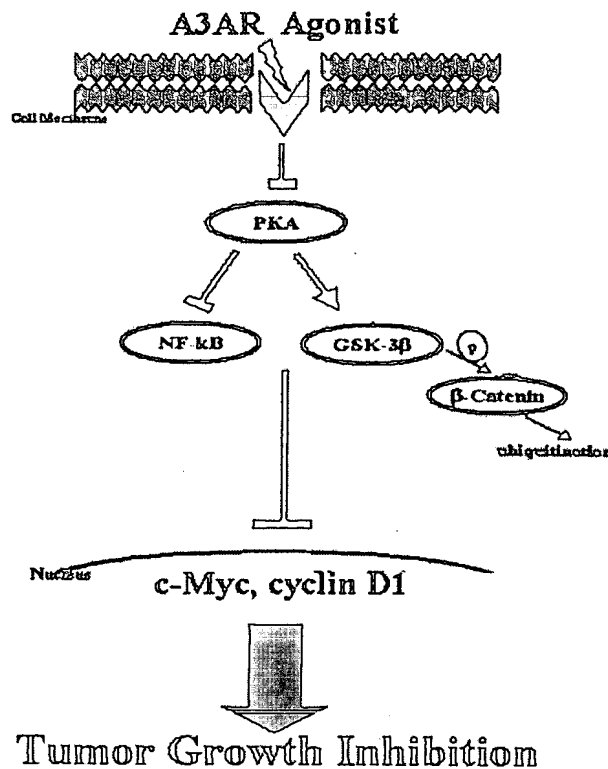


Figure 4. Schematic representation of signaling pathways that mediate A3AR inhibition of melanoma cell growth.

erodimer of p50 and p65 (Rel A) subunits in which the p65 contains the transcription activation domain. PKAc regulates the transcriptional activity of NF-κB by phosphorylating the p65 subunit of NF-κB, enabling its association with the co-activator CBP/p300 and the efficient transcriptional activity (27).

Previous reports have suggested that PC-3 prostate carcinoma cells and the androgen receptor-negative cell line (DU-145) have constitutive NF-κB activity (27, 28). Thus, the IB-MECA's capability to suppress NF-κB expression may serve as part of the mechanism through which it exerts an inhibitory effect on androgen-independent cells.

*In vivo*, the protein markers were significantly modulated upon a single or chronic exposure of the tumors to IB-MECA. One conclusion that can be drawn from this phenomenon is that these protein markers may serve as biomarkers for predicting the response of the tumor to IB-MECA in the host. These results provide a rationale to examine the protein markers in patients on IB-MECA treatment.

Collectively, these results suggest that IB-MECA inhibits the growth of prostate cancer cells *via* modulation of key proteins involved in the Wnt and NF-κB signaling pathway. Th-

ese results corroborate our findings in other types of neoplasias (melanoma and colon carcinoma) and propose the use of A3AR agonists for the management of human prostate cancer.

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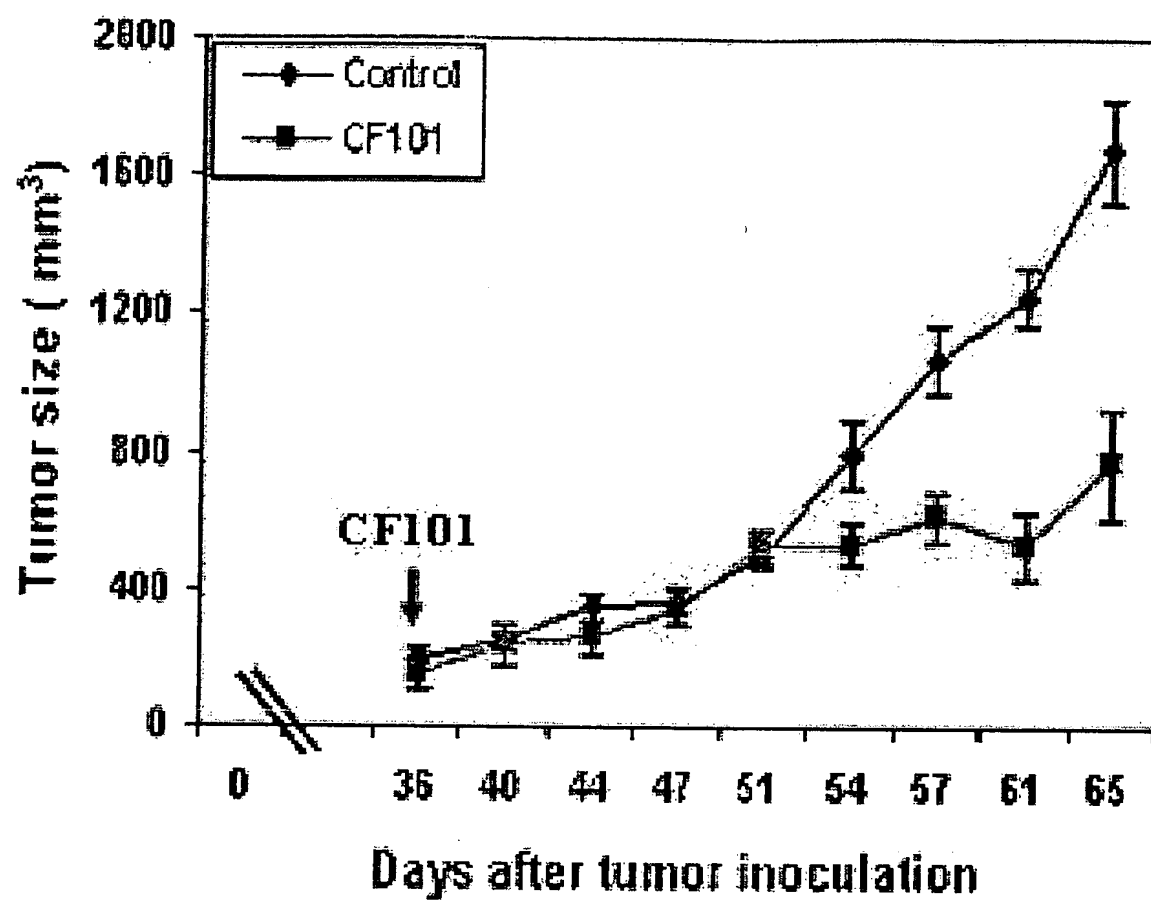


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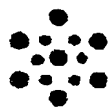
Accepted February 4, 2003

## **ANNEX C**



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## **ANNEX D**

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**From :** Gervais NELIAT, PhD  
**Date :** November 14, 2001  
**# pages :** 6  
**Ref. :** 01/GN/IM  
**Copies :**

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**To :** Dr. William KERNS  
**Company :** CANFITE BIOPHARMA (USA)  
**Fax :** (00 1) 978 456 9976

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**Subject : Study # 5367**

Dear Doctor KERNS,

Please find attached the complete results of the above mentioned study. We they will meet with your approval.

You will receive the final report very soon.

Yours sincerely,

Gervais NELIAT, PhD  
Study Director

*Prima  
FYI.  
LOOKS GREAT!  
Bill*

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Study report # 5367

p. 2/6

Table 1

IC<sub>50</sub> and K<sub>i</sub> values determined for CF101(IB-MECA)  
and the reference compounds at the receptors studied

Receptors	CF101(IB-MECA)			Reference compounds		
	IC <sub>50</sub> (nM)	K <sub>i</sub> (nM)	(nH)		IC <sub>50</sub> (nM)	K <sub>i</sub> (nM) (nH)
A <sub>1</sub> (h)	> 1,000	-	-	DPCPX	6.3	3.8 (1.0)
A <sub>2A</sub> (h)	685	560	(0.8)	NECA	44	36 (0.8)
A <sub>2B</sub> (h)	47,600	42,300	(1.1)	NECA	853	759 (0.5)
A <sub>3</sub> (h)	0.68	0.47	(0.5)	IB-MECA	1.3	0.92 (1.0)

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Study report # 5367

p. 3/6

COMPETITION CURVE OBTAINED WITH CF101(IB-MECA)  
AT THE HUMAN A1 RECEPTOR

IC50 &gt; 1000 nM

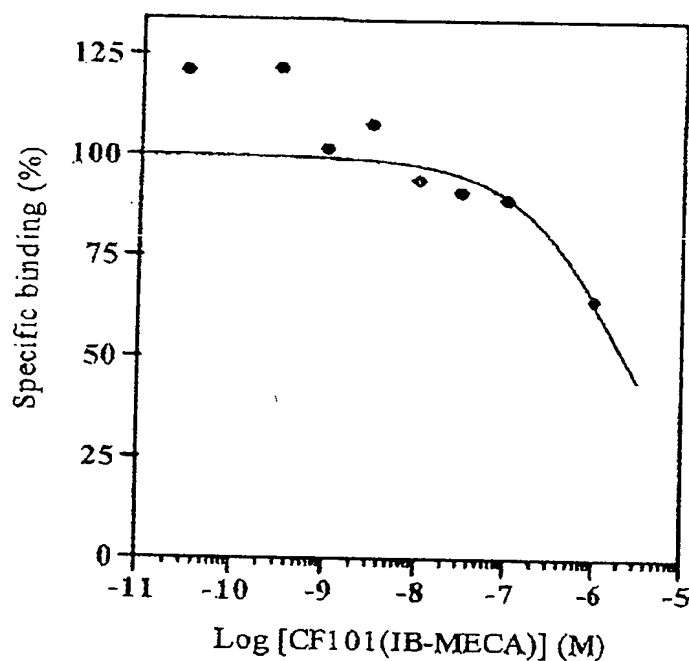


Figure 1

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Study report # 5367

p. 4/6

COMPETITION CURVE OBTAINED WITH CF101(IB-MECA)  
AT THE HUMAN A2A RECEPTOR

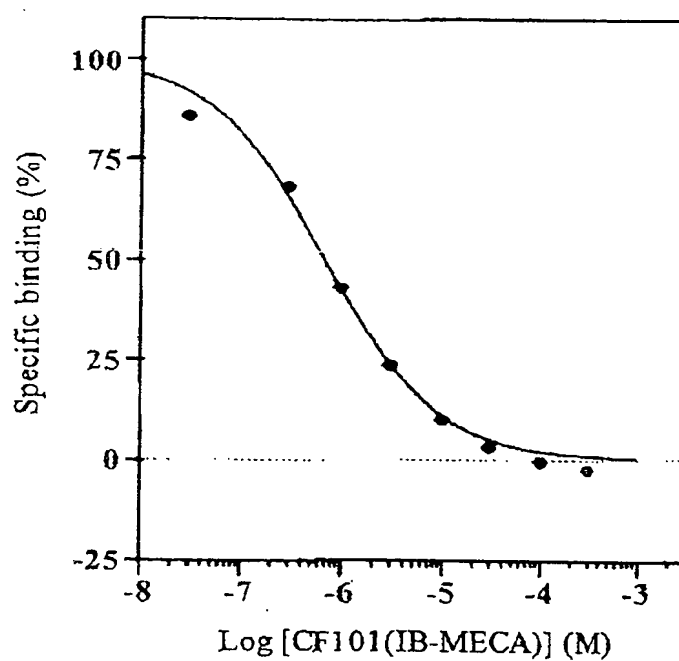
 $IC_{50} = 685 \text{ nM}$  $nH = 0.8$ 

Figure 2

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Study report # 5367

p. 5/6

COMPETITION CURVE OBTAINED WITH CF101(IB-MECA)  
AT THE HUMAN A2B RECEPTOR

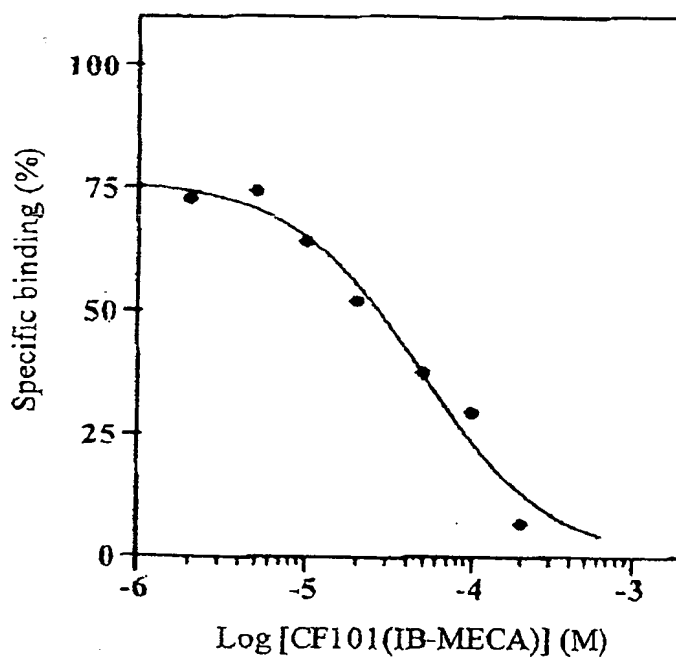
 $IC_{50} = 47600 \text{ nM}$  $nH = 1.1$ 

Figure 3

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Study report # 5367

p 6/6

COMPETITION CURVE OBTAINED WITH CF101(IB-MECA)  
AT THE HUMAN A3 RECEPTOR

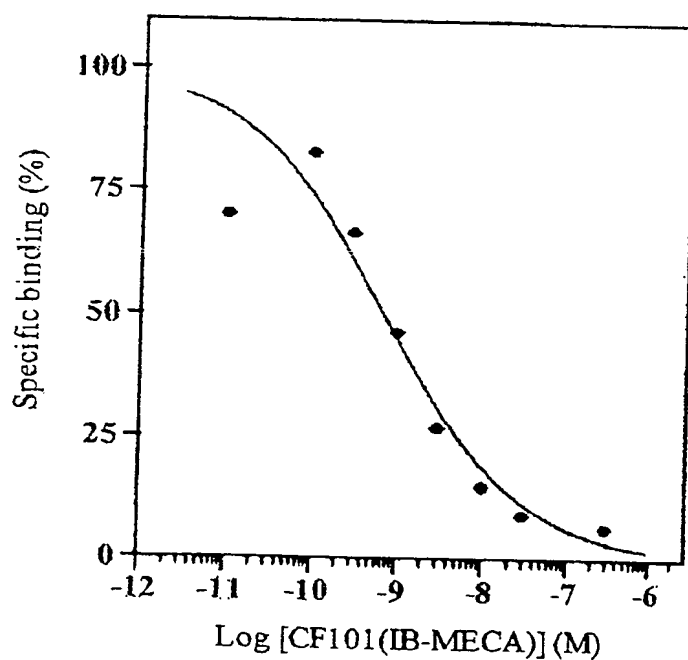
 $IC_{50} = 0.68 \text{ nM}$  $nH = 0.5$ 

Figure 4

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## **ANNEX E**

## p53-Independent induction of Fas and apoptosis in leukemic cells by an adenosine derivative, Cl-IB-MECA

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Received 26 July 2001; accepted 9 August 2001

### Abstract

A<sub>3</sub> adenosine receptor (A<sub>3</sub>AR) agonists have been reported to influence cell death and survival. The effects of an A<sub>3</sub>AR agonist, 1-[2-chloro-6-[[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-1-deoxy-N-methyl-β-D-ribofuranonamide (Cl-IB-MECA), on apoptosis in two human leukemia cell lines, HL-60 and MOLT-4, were investigated. Cl-IB-MECA (≥30 μM) increased the apoptotic fractions, as determined using fluorescence-activated cell sorting (FACS) analysis, and activated caspase 3 and poly-ADP-ribose-polymerase. Known messengers coupled to A<sub>3</sub>AR (phospholipase C and intracellular calcium) did not seem to play a role in the induction of apoptosis. Neither dantrolene nor BAPTA-AM affected the Cl-IB-MECA-induced apoptosis. Cl-IB-MECA failed to activate phospholipase C in HL-60 cells, while UTP activated it through endogenous P2Y<sub>2</sub> receptors. Induction of apoptosis during a 48 hr exposure to Cl-IB-MECA was not prevented by the A<sub>3</sub>AR antagonists [5-propyl-2-ethyl-4-propyl-3-(ethylsulfanylcabonyl)-6-phenylpyridine-5-carboxylate] (MRS 1220) or *N*-[9-chloro-2-(2-furanyl)[1,2,4]triazolo[1,5-*c*]quinazolin-5-yl]benzeneacetamide (MRS 1523). Furthermore, higher concentrations of MRS 1220, which would also antagonize A<sub>1</sub> and A<sub>2A</sub> receptors, were ineffective in preventing the apoptosis. Although Cl-IB-MECA has been shown in other systems to cause apoptosis through an A<sub>3</sub>AR-mediated mechanism, in these cells it appeared to be an adenosine receptor-independent effect, which required prolonged incubation. In both HL-60 and MOLT-4 cells, Cl-IB-MECA induced the expression of Fas, a death receptor. This induction of Fas was not dependent upon p53, because p53 is not expressed in an active form in either HL-60 or MOLT-4 cells. Cl-IB-MECA-induced apoptosis in HL-60 cells was augmented by an agonistic Fas antibody, CH-11, and this increase was suppressed by the antagonistic anti-Fas antibody ZB-4. Therefore, Cl-IB-MECA induced apoptosis *via* a novel, p53-independent up-regulation of Fas. Published by Elsevier Science Inc.

**Keywords:** Adenosine receptor; Antagonist; Poly-ADP-ribose polymerase; CD95; APO-1; Phospholipase C; HL-60; MOLT-4

### 1. Introduction

Following the introduction of A<sub>3</sub>AR-selective ligands [1–3], the A<sub>3</sub>AR has been demonstrated to have diverse physiological functions, including its effects on inflammation [4], hypotension [5], mast cell degradation [6], protection of brain and heart [7–9], and apoptosis [10–15]. Specifically, potent A<sub>3</sub>AR agonists showed dual effects leading to either cellular protection or death. In rat astroglial and human astrocytoma cells, micromolar concentrations of A<sub>3</sub>AR agonists reduced the cell number, while

nanomolar concentrations promoted cytoskeletal changes that were associated with cytoprotection [13,16]. In chick ventricular myocyte culture [14] and in the isolated rabbit heart [17], the activation of the A<sub>3</sub>AR showed a preconditioning-like effect that improved the outcome following an ischemic injury. Similar effects were observed *in vivo* in a gerbil model of global ischemia [8]. In promyelocytic human leukemia HL-60 cells [11], the A<sub>3</sub>AR agonists IB-MECA (1-[6-[[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-1-deoxy-N-methyl-β-D-ribofuranuronamide) and Cl-IB-MECA induced apoptosis at high concentrations. At lower concentrations, they protected the cells from apoptosis induced by A<sub>3</sub>AR antagonists. In CHO cells transfected with the human A<sub>3</sub>AR, effects on the cell cycle were induced with high concentrations of Cl-IB-MECA in those cells expressing the A<sub>3</sub>AR, but not in control cells [18].

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Abbreviations: A<sub>3</sub>AR, A<sub>3</sub> adenosine receptor; FACS, fluorescence-activated cell sorting; PLC, phospholipase C; PARP, poly-ADP-ribose-polymerase.

This study sought to probe the mechanism of the apoptotic cell death induced by CI-IB-MECA in the HL-60 and MOLT-4 leukemic cell lines.

## 2. Materials and methods

### 2.1. Materials

HL-60 and MOLT-4 cells were obtained from the ATCC. RPMI 1640 medium, fetal bovine serum, penicillin, streptomycin, and glutamine were purchased from Gibco BRL. CI-IB-MECA and MRS 1220 (*N*-[9-chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-*c*]quinazolin-5-yl]benzeneacetamide) were obtained from RBI-Sigma. MRS 1523 [5-propyl-2-ethyl-4-propyl-3-(ethylsulfanylcarbonyl)-6-phenylpyridine-5-carboxylate] was synthesized as described previously [19]. A TACS<sup>TM</sup> 2 TdT-DAB *in situ* apoptosis detection kit was obtained from Trevigen. Dowex AG 1-X8 resin was purchased from Bio-Rad. Anti-Fas (C20), anti-Bcl-2 (100), anti-Bax (B-9), and anti-TRAIL (C-19) were purchased from Santa Cruz Biotechnology. Anti-Fas antibodies (CH-11 and ZB-4) were purchased from Upstate Biotechnology. Anti-Cpp32 and anti-PARP (C2-10) were purchased from Pharmingen. All other reagents were purchased from Sigma.

### 2.2. Cell culture and preparation

HL-60 or MOLT-4 cells were grown at 37° in a humidified incubator with 5% CO<sub>2</sub>/95% air in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, 100 mg/mL of streptomycin, and 2 mM L-glutamine. The culture was maintained by splitting every third day. For each experiment, the cells undergoing log phase growth were collected and resuspended in growth medium to  $0.5 \times 10^6$  cells/mL, and 3 mL aliquots were placed into the individual wells of several 6-well culture plates. Test compounds dissolved in DMSO at the appropriate concentration (or only DMSO as control) were added to each well. The final concentration of DMSO in each sample did not exceed 0.2%.

### 2.3. Cell viability analysis

Cell viability was measured using the Trypan blue exclusion test. Trypan blue was mixed with the cell suspension (final concentration of dye was 0.2%), and the numbers of unstained (live) and stained (dead) cells were counted between 5 and 7 min after the dye was added.

### 2.4. Apoptosis analysis by flow cytometry

After treating HL-60 or MOLT-4 cells with various reagents, the cells were washed twice with cold PBS by centrifugation (500 g for 5 min at room temperature),

washed again with cold citrate buffer (250 mM sucrose, 5% DMSO, 40 mM trisodium citrate, pH 7.5), and fixed with 2% paraformaldehyde at 4°. The cells were washed, resuspended in 100  $\mu$ L of cold citrate buffer, and treated with 900  $\mu$ L of trypsin solution (500 units/mL of trypsin, 3.4 mM trisodium citrate, 0.1% Tergitol (type NP-40, Sigma), 1.5 mM spermine, 0.5 mM Tris (pH 7.5)) for 10 min at room temperature. Then 750  $\mu$ L of RNase solution (500  $\mu$ g/mL of trypsin inhibitor, 100  $\mu$ g/mL of RNase A, 3.4 mM trisodium citrate, 0.1% NP-40, 1.5 mM spermine, 0.5 mM Tris (pH 7.5)) was added. After a 10 min incubation at room temperature, cells were stained by the addition of 750  $\mu$ L of propidium iodide solution (0.6 mM propidium iodide, 3 mM spermine, 3.4 mM trisodium citrate, 0.1% NP-40, 0.5 mM Tris (pH 7.5)). The apoptotic fraction was quantified for  $10^4$  cells by analyzing the sub-G<sub>1</sub> (sub-diploid) population by measuring the fluorescence activity of propidium iodide-stained DNA of fixed cells on a FacsCalibur (Becton Dickinson).

### 2.5. Immunoblotting analysis

Proteins from the treated HL-60 or MOLT-4 cells were extracted using a lysis buffer (0.5% NP-40, 120 mM NaCl, 40 mM Tris (pH 8.0)). After SDS-PAGE, the protein bands were transferred to nitrocellulose paper, and were blocked with 5% powdered non-fat milk. They were incubated overnight with the primary antibodies and for 1 hr with the horseradish peroxidase linked secondary antibodies. Immunoblots were developed with enhanced chemiluminescence (ECL) reagents (Pierce).

### 2.6. Phospholipase C assay

The amount of inositol phosphates was measured by a modification of the method of Baek *et al.* [20]. After labeling with *myo*-[<sup>3</sup>H]-inositol (1  $\mu$ Ci/ $10^6$  cells) for 24 hr at 37°, LiCl was added (final concentration was 20 mM). Then the cells were resuspended to a density of  $2 \times 10^7$  cells/mL in RPMI 1640 medium containing 0.5% fetal bovine serum, 20 mM HEPES (pH 7.2), 20 mM LiCl, and 1 mg/mL of bovine serum albumin. An aliquot (150  $\mu$ L) of cell suspension was transferred to each well of a 96-well plate that contained either CI-IB-MECA or UTP. The plates were incubated for 30 min at 37°. The reaction was terminated by the addition of 100  $\mu$ L of ice-cold 180 mM formic acid. After centrifugation (2000 g for 10 min at 4–6°), the supernatants were neutralized with 300  $\mu$ L of 60 mM NH<sub>4</sub>OH and applied to Bio-Rad Dowex AG 1-X8 anion exchange columns. The columns were washed with water followed by a 60 mM sodium formate solution containing 5 mM sodium tetraborate. Total inositol phosphates were eluted with 1 M ammonium formate containing 0.1 M formic acid, and the amounts of radioactivity were measured using a liquid scintillation counter (Beckman).

## 2.7. HPLC analysis of MRS 1220 stability

To the culture medium used in the experiments, 2 vol. of acetone was added and the mixture was centrifuged (500 g for 10 min at room temperature). The supernatant was removed and concentrated under a stream of  $N_2$ . This method was shown to recover the MRS 1220 efficiently from the growth medium. The amount of MRS 1220 in the concentrate was analyzed with a Hewlett-Packard 1090 HPLC apparatus equipped with a Phenomenex<sup>®</sup> RP-C18 analytical column (250 mm  $\times$  4.6 mm, linear gradient solvent system: 0.1 M triethylammonium acetate/ $CH_3CN$  from 0/100 to 60/40 in 20 min, flow rate 1 mL/min). UV detection at 260 nm was used. Under these conditions, MRS 1220 had a retention time of 4.8 min.

## 3. Results

### 3.1. Induction of apoptosis by Cl-IB-MECA

HL-60 or MOLT-4 cells were treated with 0–30  $\mu M$  Cl-IB-MECA for 24 or 48 hr, and the fraction of cells undergoing apoptosis was determined using FACS. Significant apoptosis was induced by 30  $\mu M$  Cl-IB-MECA in both cell lines (36 and 58% in HL-60 cells, and 29 and 48% in MOLT-4 cells, at 24 and 48 hr, respectively), as indicated in Fig. 1. This apoptosis rate was confirmed using the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) method (TACS<sup>™</sup> 2 TdT-DAB *in situ* apoptosis detection kit; data not shown). The degree of cell death determined using the dye exclusion

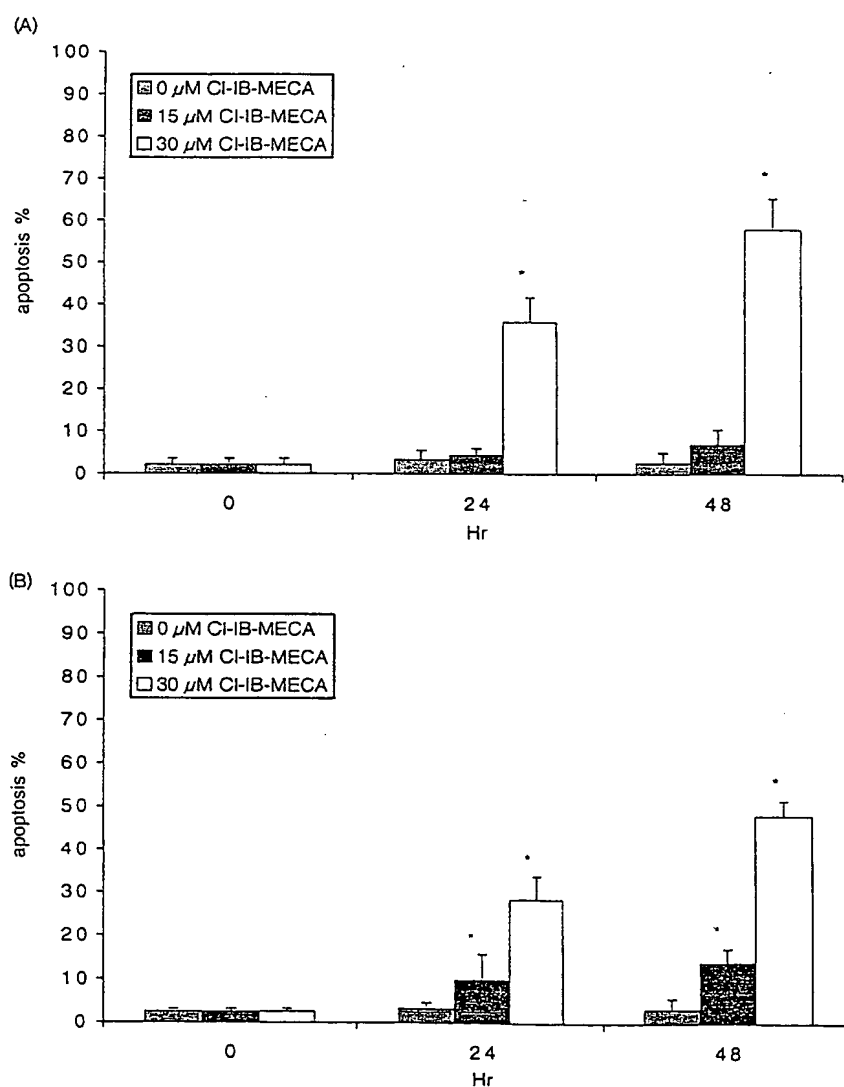


Fig. 1. Induction of apoptosis by Cl-IB-MECA in HL-60 (A) and MOLT-4 (B) cells. After treatment with 0, 15, or 30  $\mu M$  Cl-IB-MECA for 24 or 48 hr, the cells were collected and then analyzed by flow cytometry as described in Section 2. Data shown are means  $\pm$  SD of at least three independent experiments. Key: significantly different with respect to the 0 hr value, \* $P < 0.01$  (Student's *t*-test).

technique was also consistent with the degree of apoptosis. In HL-60 cells, no significant apoptosis was observed at a concentration of CI-IB-MECA of  $\leq 10 \mu\text{M}$  or at exposure times shorter than 24 hr. Therefore, a  $30 \mu\text{M}$  concentration was used to study the mechanism of apoptosis induced by CI-IB-MECA in subsequent experiments. Apoptosis was not observed at 48 hr when the cells were treated for only 4 or 8 hr with CI-IB-MECA.

### 3.2. Effects of calcium modifiers on apoptosis induced by CI-IB-MECA

The role of intracellular calcium in CI-IB-MECA-induced apoptosis was examined, since it has been reported that CI-IB-MECA increases intracellular calcium levels

[10,15]. Fig. 2 shows that the CI-IB-MECA-induced apoptosis and cell death were not affected when the release of calcium from intracellular sources was blocked by dantrolene (25 or  $50 \mu\text{M}$ ), or when the intracellular calcium was chelated following preincubation with BAPTA-AM (0.1 or  $0.2 \mu\text{M}$ ). Since the apoptosis rates were not affected by the addition of calcium modifiers, any potential change in intracellular calcium levels by CI-IB-MECA would not likely be involved in apoptosis induced by CI-IB-MECA.

### 3.3. Effects of $A_3\text{AR}$ antagonists on apoptosis induced by CI-IB-MECA

Two human  $A_3\text{AR}$ -selective antagonists, MRS 1523 (also selective in rat) and MRS 1220, were examined

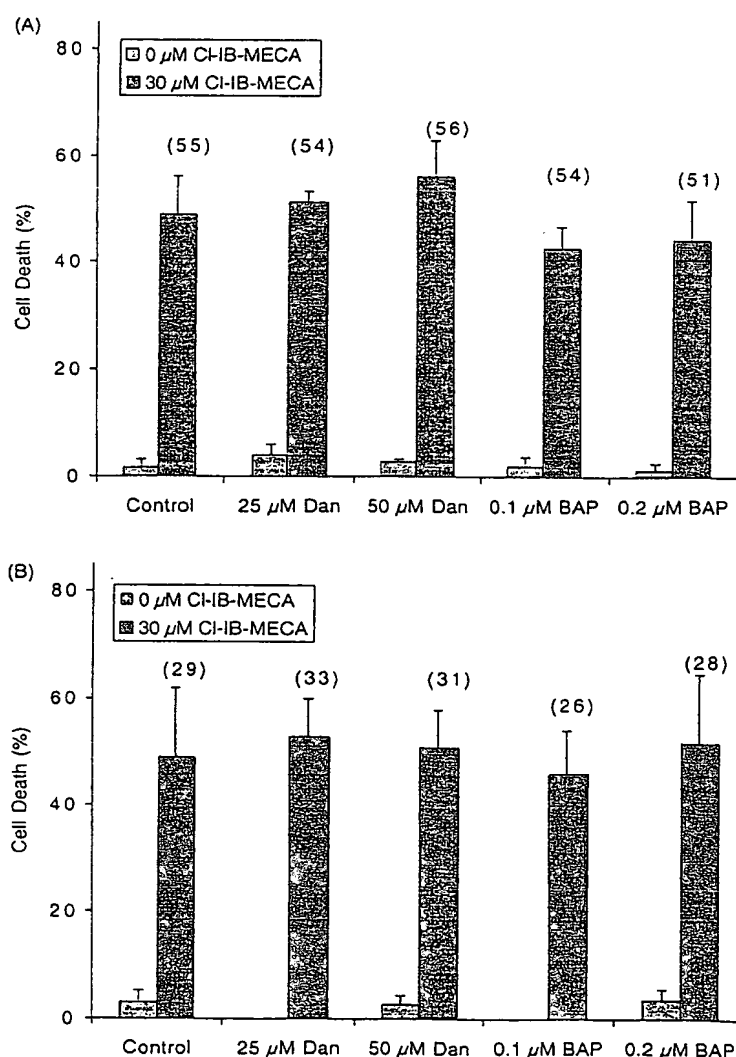


Fig. 2. Effects of calcium modifiers on CI-IB-MECA-induced cell death and apoptosis. HL-60 (A) or MOLT-4 (B) cells were incubated for 48 hr in culture medium containing calcium modulators (Dan: dantrolene, BAP: BAPTA-AM) with or without  $30 \mu\text{M}$  CI-IB-MECA. The degree of cell death was determined by the Trypan blue exclusion test. Results are means  $\pm$  SD from the combined data of two independent experiments performed in triplicate. Percent apoptosis, as determined by flow cytometry, is shown in parentheses.

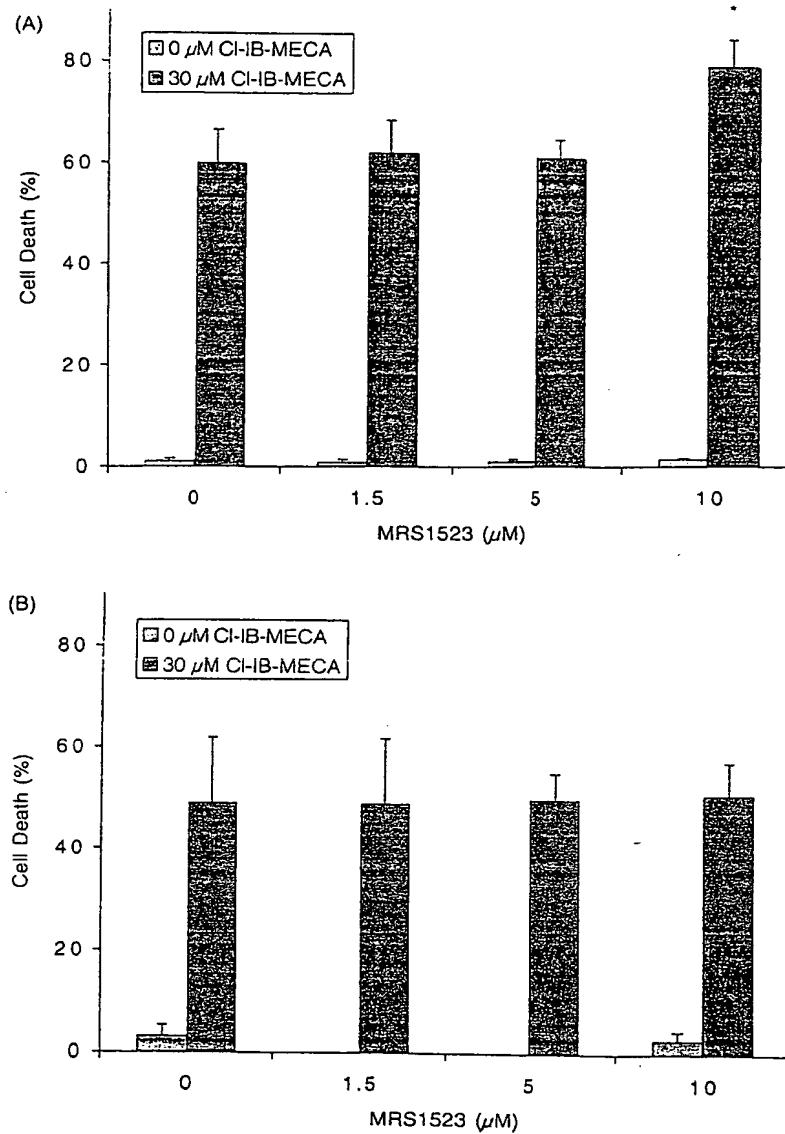


Fig. 3. Effects of the  $A_3$ AR antagonist MRS 1523 on CI-IB-MECA-induced cell death. HL-60 (A) or MOLT-4 (B) cells were incubated for 48 hr in culture medium containing 0–10  $\mu\text{M}$  MRS 1523 with or without 30  $\mu\text{M}$  CI-IB-MECA. Cell viability was determined by the Trypan blue exclusion test as described in Section 2. Data are means  $\pm$  SD of three independent experiments. Key: significantly different with respect to 0  $\mu\text{M}$  MRS 1523,  $^*P < 0.01$  (Student's *t*-test).

for their ability to block CI-IB-MECA-induced apoptosis in HL-60 and MOLT-4 cells. As shown in Fig. 3, a 48 hr incubation with MRS 1523 (0–10  $\mu\text{M}$ ) did not improve cell viability, which was reduced by 30  $\mu\text{M}$  CI-IB-MECA. At the highest concentration (10  $\mu\text{M}$ ), MRS 1523 slightly augmented the effect of 30  $\mu\text{M}$  CI-IB-MECA in HL-60 cells. MRS 1220 (0–5  $\mu\text{M}$ ) also did not affect the apoptotic rate induced by 30  $\mu\text{M}$  CI-IB-MECA (data not shown). At 5  $\mu\text{M}$ , MRS 1220 would be expected to block  $A_1$  and  $A_{2A}$  receptors [1]. Thus, the action of 30  $\mu\text{M}$  CI-IB-MECA at 48 hr appeared to be adenosine receptor-independent.

### 3.4. Phospholipase C activity

It has been reported that the  $A_3$ AR is coupled to phospholipase C (PLC) [21–23], considered one of the main second messenger systems coupled to the  $A_3$ AR. As shown in Fig. 4, however, the production of inositol phosphates was not increased by up to 60  $\mu\text{M}$  CI-IB-MECA in either HL-60 or MOLT-4 cells. To ascertain that a functional PLC system was present, we examined the effect of UTP, which activates endogenous  $P2Y_2$  nucleotide receptors in HL-60 cells. Treatment of HL-60 cells with UTP-activated PLC with an approximately 2-fold



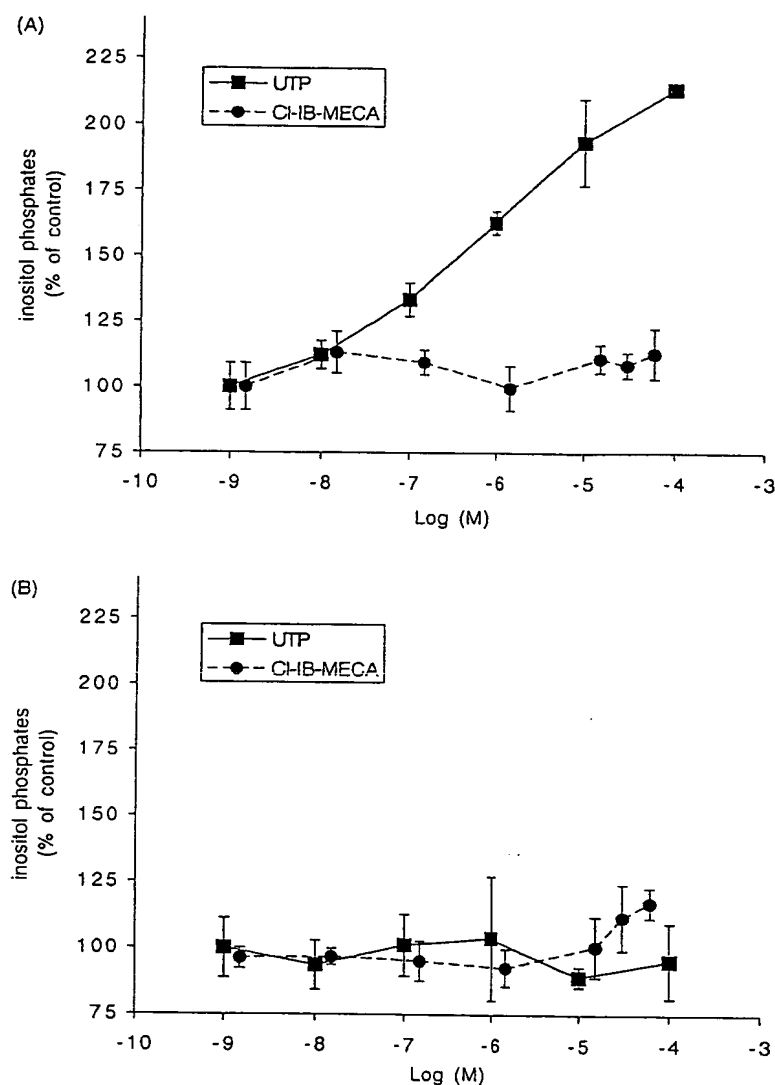


Fig. 4. Production of inositol phosphates by CI-IB-MECA. HL-60 (A) or MOLT-4 (B) cells were labeled with *myo*-[ $^3\text{H}$ ]inositol ( $1 \mu\text{Ci}/10^6$  cells) for 24 hr. Then the cells were treated for 30 min at  $37^\circ$  with CI-IB-MECA or UTP in RPMI 1640 medium containing 0.5% fetal bovine serum, 20 mM HEPES (pH 7.2), 20 mM LiCl, and 1 mg/mL of bovine serum albumin. [ $^3\text{H}$ ]inositol phosphates were extracted and separated by Dowex AG 1-X8 as described in Section 2. Results are means  $\pm$  SD from the combined data of two independent experiments performed in triplicate.

increase at  $10 \mu\text{M}$ . Therefore, HL-60 cells seemed to either lack the  $\text{A}_3\text{AR}$  or at least its ability to couple to PLC. UTP had no effect in MOLT-4 cells.

### 3.5. Western blot analysis

To understand the mechanism of apoptosis induced by CI-IB-MECA ( $30 \mu\text{M}$ ), several apoptotic biomarkers were investigated by means of immunoblotting (Fig. 5). Caspase 3 and poly-ADP-ribose-polymerase (PARP) were activated in both leukemic cell lines in a time-dependent manner, showing that CI-IB-MECA-induced apoptosis through the caspase pathway. We also observed a significant increase of Fas following 24 and 48 hr exposures to CI-IB-MECA.

There were no changes in the expression of TRAIL, Bcl-2, or Bax (data not shown).

### 3.6. Effects of agonistic (CH-11) and antagonistic (ZB-4) anti-Fas antibodies on apoptosis induced by CI-IB-MECA

Due to its pronounced up-regulation in these cell lines, the involvement of Fas expression in CI-IB-MECA-induced apoptosis was investigated further. HL-60 (Fig. 6A) and MOLT-4 (Fig. 6B) cells were treated for 24 hr with CI-IB-MECA and/or CH-11, an agonistic Fas antibody [24], and apoptosis was determined using FACS. The apoptosis rate in both cell lines was not changed significantly by CH-11 alone during 24 hr, although there

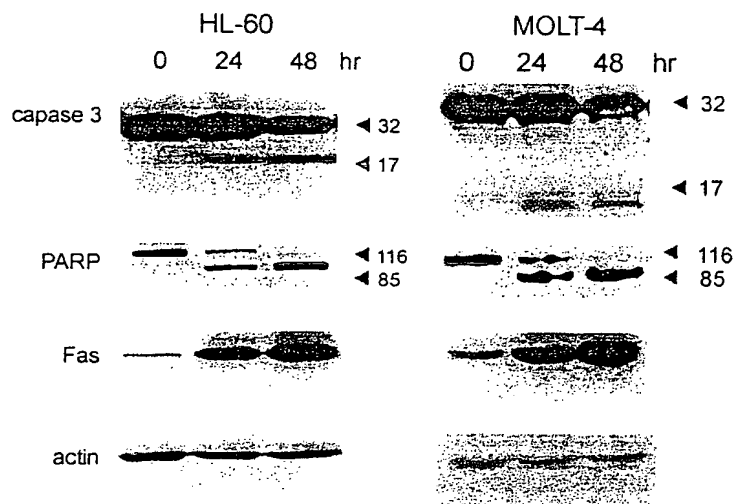


Fig. 5. Activation of caspase 3 and PARP and increase of Fas expression in HL-60 and MOLT-4 cells. After treating cells with 30  $\mu$ M Cl-IB-MECA for 24 and 48 hr, proteins were extracted, separated, and analyzed by Western blotting as described in Section 2. Antibody detection of actin bands demonstrated that the same amount of protein was present in each lane. Blots shown are representative of at least three independent experiments.

were slight increases of cell death at 1  $\mu$ g/mL, using the Trypan blue exclusion test (data not shown). Apoptosis induced by Cl-IB-MECA (30  $\mu$ M) in HL-60 cells (Fig. 6A) was augmented to 130 and 180% of control with 0.25 and 1.0  $\mu$ g/mL of CH-11, respectively. Augmentation effects by Cl-IB-MECA and subthreshold doses of CH-11 were observed in MOLT-4 cells (Fig. 6B). A Fas antagonist was also used to demonstrate that this increase in Cl-IB-MECA-induced apoptosis elicited by CH-11 was dependent upon Fas. The pretreatment of HL-60 cell cultures with the Fas antagonistic antibody ZB-4 (4 or 8  $\mu$ g/mL) for 1 hr, and its continued presence during the subsequent incubation, reduced the degree of apoptosis induced by prolonged (24 hr) exposure to Cl-IB-MECA (30  $\mu$ M) and CH-11 (1  $\mu$ g/mL) to the control level of approximately 30% (data not shown). These results implied that increased Fas expression mediated the apoptotic effect of Cl-IB-MECA.

#### 4. Discussion

It has been reported that  $A_3$ AR agonists induce cell death in leukemic and other cells [10–15], while the mechanisms remain unknown. The present study was undertaken to determine how Cl-IB-MECA, an  $A_3$ AR agonist, induced apoptosis in leukemic cells.

Intracellular calcium has been known to modulate or transduce many intracellular signals including those of programmed cell death or apoptosis. The increase in intracellular free calcium following the activation of the  $A_3$ AR has been reported in HL-60 cells and in cardiac myocytes [10,15]. We have investigated whether Cl-IB-MECA caused apoptotic cell death through an increase of

intracellular calcium levels. As shown in Fig. 2, the induction of cell death by Cl-IB-MECA was not affected when the calcium release from sarcoplasmic reticulum was inhibited by dantrolene, or when the free intracellular calcium was removed by chelation. Therefore, the change of intracellular calcium did not seem to play a role in the Cl-IB-MECA-induced apoptosis.

The  $A_3$ AR increases intracellular calcium levels through the coupling to PLC [1]. Therefore, we tested the activity of PLC in these leukemic cells upon treatment with Cl-IB-MECA (Fig. 4). Inositol phosphates were not increased by Cl-IB-MECA in either HL-60 or MOLT-4 cells. However, inositol phosphates were increased by UTP used as a positive control that acts through  $P2Y_2$  receptors known to be expressed in HL-60 cells (Fig. 4). When UTP was added to HL-60 cells, it increased the production of inositol phosphates in a concentration-dependent manner, demonstrating the existence of purinergic receptors coupled to PLC. However, MOLT-4 cells appear to lack  $P2Y_2$  or  $P2Y_4$  receptors, which are normally activated by UTP, although the presence of  $P2Y_6$  receptors in MOLT-4 cells has been reported [25]. Therefore, Cl-IB-MECA did not act through a PLC-coupled  $A_3$ AR.

Additional evidence that the apoptotic effect of Cl-IB-MECA was not exerted through  $A_3$ AR activation was obtained using the selective  $A_3$ AR antagonist MRS 1523. The presence of this antagonist in the medium throughout the incubation did not block or diminish the Cl-IB-MECA-induced cell death (Fig. 3). Another antagonist of adenosine receptors, MRS 1220 (at 1 and 5  $\mu$ M), also had no effect (data not shown). Since no antagonism was observed, the stability of MRS 1220 in the experimental conditions was investigated. It was demonstrated by HPLC analysis that MRS 1220 remained intact during a

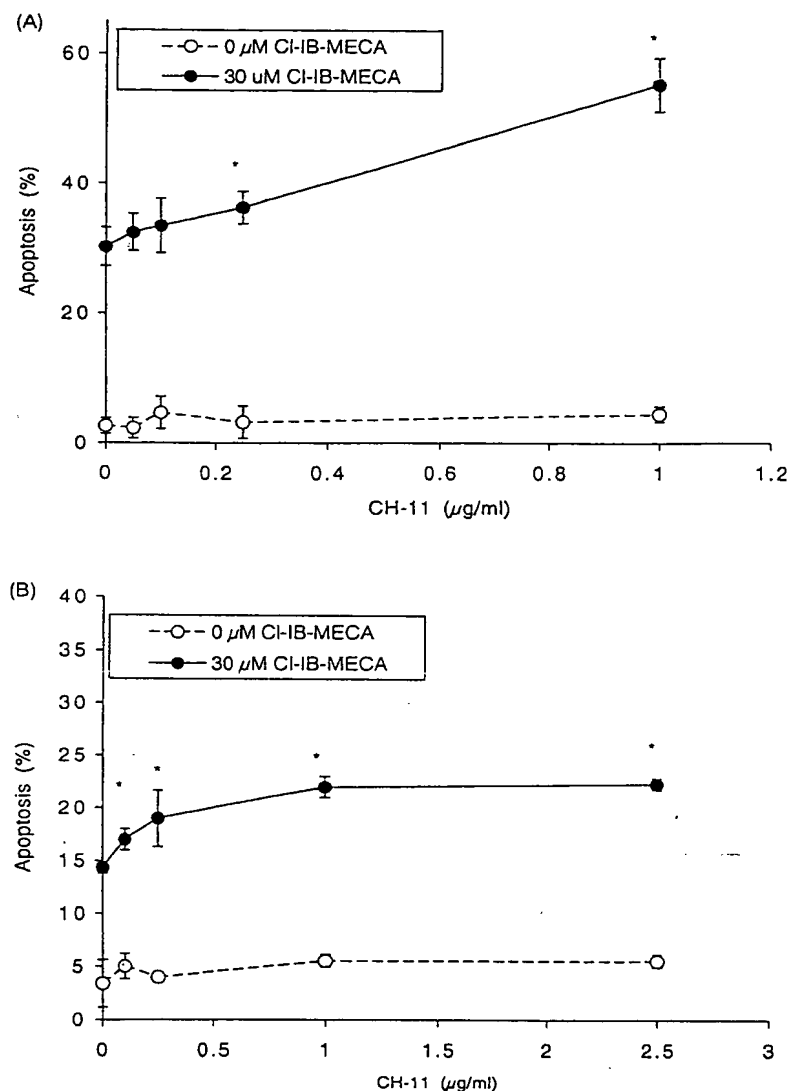


Fig. 6. Effect of CH-11 on Cl-IB-MECA-induced apoptosis. HL-60 (A) or MOLT-4 (B) cells were incubated for 24 hr in culture medium containing various concentrations of CH-11 with or without 30  $\mu$ M Cl-IB-MECA. The apoptotic rate was determined by the Trypan blue exclusion test and by flow cytometry as described in Section 2. Data are means  $\pm$  SD of three independent experiments. Key: significantly different with respect to 0  $\mu$ g/mL of CH-11, \* $P$  < 0.01 (Student's  $t$ -test).

48 hr incubation in the presence of HL-60 cells (data not shown). Therefore, a mechanism other than activation of adenosine receptors seems to be responsible. In rat cardiac myocytes [26], a lower concentration of antagonist (MRS 1523, 1  $\mu$ M) was reported to antagonize apoptosis induced by 20  $\mu$ M IB-MECA, but only when the agonist was present for a short period, i.e. 2 hr. In the leukemic cells, however, we observed that the short periods of treatment with antagonists (2, 4.5, 9, 18 hr) also did not prevent the apoptosis induced by Cl-IB-MECA (24 hr). Under conditions similar to those of Fig. 1, a newly reported  $A_3$  agonist [27], MRS 1898 (30  $\mu$ M), did not induce apoptosis significantly (5% at 48 hr compared with 60% for Cl-IB-MECA), which is additional evidence that the Cl-IB-

MECA-induced apoptosis in HL-60 cells does not occur through the  $A_3$ AR (data not shown). Nevertheless, the presence of  $A_3$ AR mRNA in HL-60 cells has been shown [10] by reverse transcription-polymerase chain reaction (RT-PCR). It was not possible, using  $^{125}$ I-AB-MECA, to detect radioligand binding to the  $A_3$ AR in HL-60 cell membranes [10]. There may be an insufficient amount of  $A_3$ AR on the cell membrane to transduce the signal. Cl-IB-MECA may induce apoptosis through a pathway different from  $A_3$ AR activation. However, there is also a possibility that the  $A_3$ AR may contribute to apoptosis by activating a pathway different from the second messenger signals tested above or that prolonged agonist exposure may have desensitized the  $A_3$ AR. The G proteins that

function in coupling to the A<sub>3</sub>AR may be involved in the apoptotic process. For example, the activation of Akt, which leads to an anti-apoptotic cascade, by the m1 or m2 muscarinic receptors, both of which are G protein-coupled receptors, seems to be signaled through G $\alpha_q$ , G $\alpha_i$ , and  $\beta\gamma$  without activation of protein kinase C (PKC) [28].

When apoptosis was induced in HL-60 and MOLT-4 cells by Cl-IB-MECA, activation of caspase 3 and PARP was observed (Fig. 5). There was also an increase of Fas expression, which may mediate the apoptotic cell death (Fig. 5). Fas induction was not observed when apoptosis was induced by 10  $\mu$ M camptothecin, a topoisomerase I inhibitor, for 5 hr (data not shown). Thus, Cl-IB-MECA-induced apoptosis occurred through a pathway different from camptothecin, and the activation of the Fas signal is an important step in the pathway.

Fas, a member of the tumor necrosis factor (TNF) receptor family, has been considered to have an important role in the regulation of death in many cell types, especially in mediating signals to induce lymphocytic apoptosis and to prevent autoimmune disease [29,30]. Liver cells infected with hepatitis C virus showed up-regulation of Fas, which is thought to be activated by the Fas ligand (FasL) on the T cells in an inflamed lesion of the liver [31]. It was also suggested that the induction of Fas expression may be one of the mechanisms of action of chemotoxic drugs and thus might enhance the cell susceptibility to Fas-mediated apoptosis [32].

The importance of p53 in Fas expression has been reported recently. In human vascular smooth muscle cells, p53 activation transiently increased surface Fas expression [33]. Fas was up-regulated in p53<sup>+/+</sup>, but not in p53<sup>-/-</sup> human leukemic cells when apoptosis was induced by irradiation [34,35]. The transfection of wild-type p53 into a p53-null adenocarcinoma induced the marked up-regulation of Fas [36]. In human colon cancer cell lines, the induction of Fas by 5-fluorouracil and leucovorin was also found to be p53-dependent [37]. It was suggested that Fas enhanced p53-mediated apoptosis [38], and that p53 sensitized cells to Fas-induced apoptosis [33]. According to the data that we have shown, the increased expression of Fas by Cl-IB-MECA seems to be a novel mechanism for the induction of apoptosis in human leukemia cells, which do not have functional p53. HL-60 cells are known to be p53-null [39], and MOLT-4 cells have a mutated p53 that cannot be expressed [40]. Therefore, our results suggest a deviation from previously characterized pathways of apoptosis. It would be intriguing to understand how Cl-IB-MECA induced the expression of Fas and if the pathway is related to that of p53 signals. Although it is uncertain how Cl-IB-MECA induced the expression of Fas in these cells, we have shown that Fas can be up-regulated independently of p53.

Since Fas-mediating signaling plays an important part in the regulation of lymphocyte populations, the intervention by bisindolmaleimide VIII, which facilitates Fas-induced

apoptosis signaling processes, was suggested as a possible strategy for the treatment of autoimmune disease, by enhancing apoptosis to eliminate self-reactive T cells [41]. Widely used anticancer drugs, such as methotrexate and doxorubicin, have been known to act through the up-regulation of FasL expression, leading to apoptosis of Fas-sensitive tumor cells [42]. Here we suggest that the up-regulated Fas expression by Cl-IB-MECA may also be a good candidate strategy for autoimmune disease or for cancer treatment in combinations with those applications.

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## **ANNEX F**

## A3 Adenosine Receptor Activation in Melanoma Cells

## ASSOCIATION BETWEEN RECEPTOR FATE AND TUMOR GROWTH INHIBITION\*

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AQ: A

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From the †Can-Fite BioPharma Ltd., Kiryat-Matalon, Petach-Tikva, 49170, and the §Laboratory of Clinical and Tumor Immunology, The Felsenstein Medical Research Center, Tel-Aviv University Sackler Faculty of Medicine, Rabin Medical Center, Petach-Tikva 49100, Israel

AQ: B Activation of the G<sub>i</sub> protein-coupled A3 adenosine receptor (A3AR) has been implicated in the inhibition of melanoma cell growth by deregulating protein kinase A and key components of the Wnt signaling pathway. Receptor activation results in internalization/recycling events that play an important role in turning on/off receptor-mediated signal transduction pathways. Thus, we hereby examined the association between receptor fate, receptor functionality, and tumor growth inhibition upon activation with the agonist 1-deoxy-1-[6-[(3-iodophenyl)methyl]amino]-9H-purine-9-yl]-N-methyl-β-D-ribofuranuronamide (IB-MECA). Results showed that melanoma cells highly expressed A3AR on the cell surface, which was rapidly internalized to the cytosol and “sorted” to the endosomes for recycling and to the lysosomes for degradation. Receptor distribution in the lysosomes was consistent with the down-regulation of receptor protein expression and was followed by mRNA and protein resynthesis. At each stage, receptor functionality was evidenced by the modulation in cAMP level and the downstream effectors protein kinase A, glycogen synthase kinase-3β, c-Myc, and cyclin D1. The A3AR antagonist MRS 1523 counteracted the internalization process as well as the modulation in the expression of the signaling proteins, demonstrating that the responses are A3AR-mediated. Supporting this notion are the *in vivo* studies showing tumor growth inhibition upon IB-MECA treatment and reverse of this response when IB-MECA was given in combination with MRS 1523. In addition, in melanoma tumor lesions derived from IB-MECA-treated mice, the expression level A3AR and the downstream key signaling proteins were modulated in the same pattern as was seen *in vitro*. Altogether, our observations tie the fate of A3AR to modulation of downstream molecular mechanisms leading to

AQ: C tumor growth inhibition both *in vitro* and *in vivo*.

The incidence of melanoma in humans has increased steadily over the past years and is one of the more difficult neoplasias to clinically manage. Due to the limited response of malignant melanoma to conventional chemotherapy and the poor prognosis of patients with metastatic melanoma, new therapies for this disease are needed.

Fn1 Our earlier studies demonstrated that IB-MECA,<sup>1</sup> a stable

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AQ: X ¶ To whom correspondence should be addressed. Tel.: 972-3-9241114; Fax: 972-3-9249378; E-mail: pfishman@post.tau.ac.il.

<sup>1</sup> The abbreviations used are: IB-MECA, 1-deoxy-1-[6-[(3-

agonist to A3AR, inhibits the proliferation of neoplastic cells including metastatic melanoma (1–3). A3AR belongs to the family of the G<sub>i</sub> protein-associated cell surface receptors. Receptor activation leads to inhibition of adenylyl cyclase activity, cAMP formation, and PKA expression. PKA contains a catalytic subunit, PKAc, which dissociates from the parent molecule upon activation with cAMP, resulting in the initiation of various signaling pathways (4, 5). Recent studies have demonstrated that PKAc phosphorylates and inactivates GSK-3β (6). We showed that IB-MECA alters the expression of GSK-3β and β-catenin, key components of the Wnt signaling pathway. Consequently it led to inhibition in the expression of the cell cycle progression genes, *c-myc* and cyclin D1 (2). This is an important observation as the Wnt pathway has been linked to the development of malignant melanoma (7–9).

It is well established that G<sub>i</sub> protein receptors are internalized to early endosomes upon agonist binding. Early endosomes serve as the major site of receptor recycling, whereas the late endosomes are involved in the delivery of the internalized receptor to the lysosomes (10). One point to consider while targeting chronically a G<sub>i</sub> protein receptor is that desensitization may lead to loss of a functional receptor from the cell surface.

Interestingly, although A3AR expression level was found to be low in most body tissues, it is highly expressed in tumor cell lines (11–13). Given that IB-MECA inhibits the growth of B16-F10 melanoma cells, it was hypothesized that these cells exhibit high receptor levels, which may serve as a target for tumor growth inhibition. We thus sought to explore the fate of A3AR upon IB-MECA activation and the consequences on the downstream molecular mechanisms leading to tumor growth inhibition both *in vitro* and *in vivo*.

Here we show that melanoma cells highly express A3AR, which upon IB-MECA stimulation rapidly internalizes to the cytosol and sorts to endosomes and lysosomes. Resynthesis and externalization of the receptor to the cell surface then occurs. Receptor functionality was demonstrated by the initiation of signal transduction pathways, which resulted in down-regulation of c-Myc and cyclin D1, leading to tumor growth suppression.

## EXPERIMENTAL PROCEDURES

Reagents—IB-MECA and MRS 1523 were purchased from RBI/Sigma. For both reagents, a stock solution of 10 mM was prepared in Me<sub>2</sub>SO, and further dilutions in RPMI medium were performed. RPMI, fetal bovine serum, and antibiotics for cell cultures were obtained from

iodophenyl)methyl]amino]-9H-purine-9-yl]-N-methyl-β-D-ribofuranuronamide; AB-MECA, □□; A3AR, A3 adenosine receptor; GSK-3β, glycogen synthase kinase-3β; PBS, phosphate-buffered saline; PKA, protein kinase A; PKB, protein kinase B; FITC, fluorescein isothiocyanate.

**AQ: I** Beit Haemek, Haifa, Israel. [<sup>125</sup>I]AB-MECA was purchased from Amersham Biosciences. Rabbit polyclonal antibodies against murine and human A3AR, PKAc, c-Myc, and GSK-3 $\beta$  were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA. Rabbit polyclonal antibodies against murine and human cyclin D1 were purchased from Upstate Biotechnology, Lake Placid, NY, and Cy3-conjugated anti-goat IgG and fluorescein-conjugated anti-rabbit IgG were purchased from Chemicon, Temecula, CA. FITC-dextran, FITC-transferrin, and forskolin were obtained from Sigma, and 8-Bromo-cAMP and MG132 were obtained from Calbiochem.

**AQ: J** **Immunostaining and Confocal Microscopy**—B16-F10 murine melanoma cells were grown for 24 h on coverslips coated with poly(L-lysine) (500  $\mu$ g/ml). Cells were incubated with IB-MECA (10 nM) or with IB-MECA + MRS 1523 (100 nM). To further show the immunostaining specificity, splenocytes derived from wild type C57BL/6J mice or A3AR $^{-/-}$  mice (14) (kindly supplied by Marlene Jacobson from Merck Research Laboratories) were mounted on poly(L-lysine) slides for 3 h. Cells were fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 1 h at room temperature. The fixed cells were rinsed three times for 1 min with PBS. To block nonspecific interaction of the antibodies, cells were incubated for 30 min in 4% normal goat serum in PBS (1% bovine serum albumin, 0.1% Triton X-100). For A3AR labeling, cells were then incubated with the primary antibody against A3AR at a dilution of 1:1000 in PBS (1% bovine serum albumin, 1% normal goat serum, 0.1% Triton X-100) for 24 h at 4 °C. After being washed three times for 3 min with PBS, cells were incubated with Cy3-conjugated anti-goat IgG at a dilution of 1:250 in PBS and incubated in the dark for 2 h. Cells were rinsed with PBS three more times and mounted with AM 100 media (Chemicon, Temecula, CA). For the colocalization experiments, the endosomes were labeled by incubating cells with 200  $\mu$ g/ml FITC-transferrin in media lacking serum for 60 min before incubating with IB-MECA (10 nM) for different time periods.

**AQ: K** Lysosomes were labeled by incubating cells with 1 mg/ml FITC-dextran in RPMI with 1% serum at 37 °C for 24 h. Cells were washed with media and reincubated for an additional 1.5 h in media lacking serum following incubation with IB-MECA (10 nM) for different time periods. Cells were washed with PBS and fixed with 4% formaldehyde, and the A3AR was labeled as mentioned above. Stained cells were visualized by a confocal microscope (Zeiss, Axiovert 100 M, excitation at 553 and emission at 568 nm for Cy3, and at 492 and 520 nm, respectively, for fluorescein).

**AQ: L** **Measurement of cAMP Production**—B16-F10 melanoma cells ( $1 \times 10^6$ /ml) were serum-starved overnight and then incubated with IB-MECA. cAMP levels were determined under basal conditions and in cells challenged for 5, 15, and 30 min with forskolin (50 nM) in the presence or absence of IB-MECA (10 nM). Cells were lysed by the addition of 0.1 M HCl, and cell lysates were collected by centrifugation for 10 min at 1000 rpm. Dried samples were stored at -20 °C until used. For determination of cAMP production, a commercial enzyme-linked immunosorbent assay kit based on competitive protein binding method (R&D systems, Minneapolis, MN) was used. Four different experiments were performed.

**AQ: M** [<sup>125</sup>I]AB-MECA Cell Surface Binding—To evaluate receptor surface density upon IB-MECA treatment, a radioligand binding assay was carried out in intact B16-F10 melanoma cells (see Ref. 20). Cells were serum-starved overnight, washed with PBS, and then incubated with IB-MECA (10 nM) for different time periods at 37 °C. At the end of the incubation period, cells were placed on ice and then rapidly washed three times with 120 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, 50 mM Tris, and 1 mM EDTA, pH 3.5 (acid T<sub>1</sub> buffer) (to remove the agonist). Cells were then incubated with 0.5 nM [<sup>125</sup>I]AB-MECA in T<sub>1</sub> buffer at pH 8.12 at 4 °C for 120 min. The assay was performed in the absence or in the presence of 100 nM IB-MECA for nonspecific binding determination. This experiment was repeated three times.

**AQ: N** **Western Blot Analysis**—To detect the level of expression of the desired proteins in B16-F10 melanoma cells, Western blot analysis was performed. Cells were serum-starved overnight and then incubated in the presence and absence of IB-MECA (10 nM), MRS 1523 (100 nM), forskolin (50 nM), or MG132 (20 nM) for different time periods at 37 °C with 1% fetal bovine serum. Cells were then rinsed with ice-cold PBS and transferred to ice-cold lysis buffer (TNN buffer, 50 mM Tris buffer, pH 7.5, 150 mM NaCl, Nonidet P-40 0.5% for 20 min). Cell debris was removed by centrifugation for 10 min at 7500  $\times$  g. The supernatant was utilized for Western blot analysis. Protein concentrations were determined using the Bio-Rad protein assay dye reagent. Equal amounts of the sample (50  $\mu$ g) were separated by SDS-PAGE, using 12% polyacrylamide gels. The resolved proteins were then electroblotted onto nitro-

cellulose membranes (Schleicher & Schuell). Membranes were blocked with 1% bovine serum albumin and incubated with the desired primary antibody (dilution 1:1000) for 24 h at 4 °C.

To evaluate the specific binding, a blocking peptide corresponding to the peptide antigen (Santa Cruz Biotechnology) was used. Blots were then washed and incubated with a secondary antibody for 1 h at room temperature. Bands were recorded using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) color development kit (Promega, Madison, WI). The optical density of the bands was quantified using an image analysis system and corrected by the optical density of the corresponding actin bands. Data presented in the different figures are representative of at least three different experiments.

**Northern Blot Analysis**—Total RNA was isolated from B16-F10 melanoma cells treated with IB-MECA (10 nM) or IB-MECA + MRS 1523 (100 nM) for 1 h, utilizing TRI reagent (Sigma). The samples were then subjected twice to phenol:chloroform extraction and washed with chloroform. RNA was precipitated with sodium acetate/ethanol following washing with ethanol, and then denatured, separated (25  $\mu$ g/lane) in 1.1% formaldehyde agarose gel, and transferred to Hybond-N membrane. The 390-bp EcoRI fragment from A3AR cDNA clone of mouse (TAA31.S), kindly supplied by Dr Kathia Ravid, was prepared by random-primed synthesis. Probes were used in RNA blot analysis at a hybridization temperature of 42 °C in the presence of 50% formamide.

**In Vivo Studies**—C57BL/6J, male mice (Harlan Laboratories, Jerusalem, Israel) aged 2 months, weighing an average of 25 g, were used. Mice were maintained on a standardized pelleted diet and supplied with tap water. Experiments were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at the Rabin Medical Center, Petah Tikva, Israel.

The effect of IB-MECA on the development of subcutaneous tumors in C57BL/6J mice was studied. B16-F10 ( $2.5 \times 10^5$ ) melanoma cells were subcutaneously injected to mice flank. Treatments as detailed below were administered orally twice daily, starting 24 h after the inoculation of the tumor cells. Four groups of mice were included in the study and treated as follows: 1) control, vehicle only; 2) IB-MECA, 10  $\mu$ g/kg; 3) IB-MECA (10  $\mu$ g/kg) + MRS 1523 (100  $\mu$ g/kg); 4) MRS 1523, 100  $\mu$ g/kg.

On day 15, the mice were treated with IB-MECA and sacrificed after 1 h. Tumor size (width (W) and length (L)) was measured with a caliper and calculated according to the following formula: tumor Size = (W)<sup>2</sup>  $\times$  L/2. Tumor lesions were then excised and homogenized (Polytron, K1-NEMATICA), and protein was extracted and subjected to Western blot analysis for the determination of A3AR. Each group contained 15 mice, and the study was repeated three times.

**Statistical Analysis**—The results were evaluated using the Student's *t* test, with statistical significance at *p* < 0.05. Comparison between the mean value of different experiments was carried out

## RESULTS

**Localization of A3AR in B16-F10 Melanoma Cells**—To study receptor localization, we utilized confocal laser microscopy. Untreated cells (control) highly exhibited A3AR on the cell surface, as seen from the fluorescence intensity level. A marked decrease in the fluorescence level was noted after 5 min in the IB-MECA-treated cells. Exposure of the melanoma cells to the antagonist MRS 1523 in the presence of IB-MECA resulted in cell surface fluorescence intensity similar to that of the control (Fig. 1A). These data suggest that rapid receptor internalization took place upon IB-MECA treatment. The specificity of receptor immunostaining was evidenced by showing marked fluorescence in splenocytes derived from wild type mice as compared with negative staining in splenocytes from A3AR knockout mice (Fig. 1B).

To further explore the time course kinetic of A3AR internalization, B16-F10 melanoma cells were exposed for different time periods to IB-MECA, and confocal microscopy analysis was carried out. Fig. 2 depicts the gradual internalization rate that occurred within a few minutes, resulting in the disappearance of the fluorescence after 6 min. Prolonged exposure (15 min) of the melanoma cells to IB-MECA resulted in receptor recycling to the cell surface. This was followed by internalization/recycling after longer incubation time periods (30 and 60 min). To confirm the observation that the fluorescence level is decreased as a result of internalization, we performed optical

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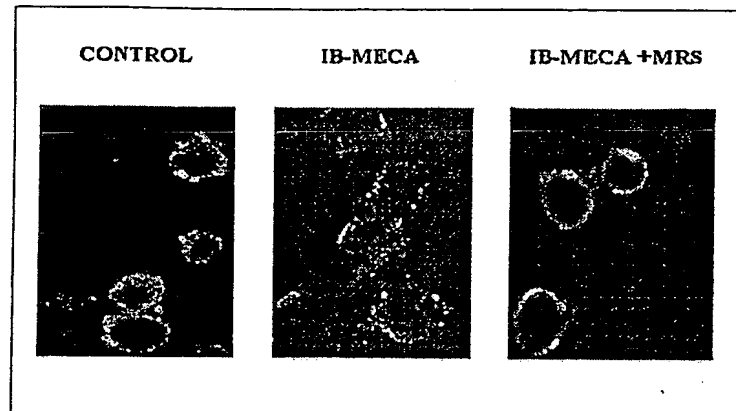
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## Modulation of A3 Adenosine Receptor in Melanoma Cells

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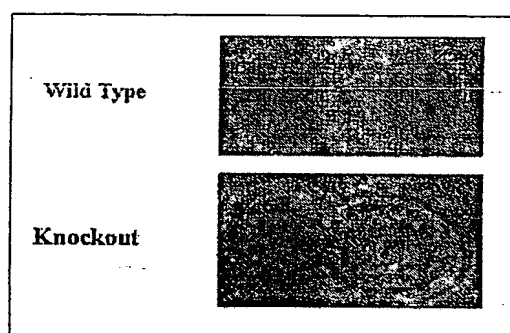


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FIG. 1. Melanoma cells highly exhibit A3AR, which is down-regulated upon IB-MECA treatment (confocal microscopy imaging). B16-F10 melanoma cells were incubated for 5 min at 37 °C with 10 nM IB-MECA. The cells were labeled with the primary and secondary antibodies against A3AR and the Cy3-conjugated anti-goat IgG, respectively. Images represent the center section of the X-Y plane. A, exhibition of A3AR in melanoma cells. High fluorescence intensity is depicted in the control cells, whereas in IB-MECA-treated cells, lower fluorescence is seen. The combined treatment with IB-MECA and the antagonist MRS 1523 (100 nM) for 5 min results in fluorescence similar to that of the control. B, splenocytes derived from wild type mice (showing A3AR-positive staining) in comparison with splenocytes derived from A3AR knockout mice (negative staining).

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sectioning of the cells. In untreated cells (control), the receptor was exhibited on the cell surface (Fig. 3, upper left), and upon exposure to IB-MECA for 5 min, it was presented inside the cell (Fig. 3, upper right), supporting the notion that A3AR translocates from the membrane to the cytosol. After 15 and 60 min, the receptor was accumulated in the cytosol (Fig. 3, lower left and right).

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To assess differences in subcellular localization of A3AR following exposure to IB-MECA, we examined the time-dependent colocalization of A3AR with FITC-transferrin and FITC-dextran, known to accumulate in distinct subcellular compartments. Fig. 4A demonstrates that in unstimulated cells, the A3AR distribution (green) displays a membrane localization pattern, whereas transferrin (red) was accumulated in small vesicles. At 5 and 15 min after treatment with IB-MECA, a significant colocalization of A3AR and transferrin in the early endosomes was observed (colocalization shown in yellow/orange). However, when cells were exposed to IB-MECA for 60 min, less colocalization of A3AR and transferrin was evident (Fig. 4A). Time-dependent localization of A3AR with lysosomes (red) was revealed in cells labeled with FITC-dextran. Unstimulated cells displayed A3AR on the cell surface, and dextran was localized to large vesicles typical of lysosomes, many of which are centrally located in the cells (Fig. 4B, upper left). After 5 min of incubation with IB-MECA, some colocalization with dextran was observed. Following 15 min of exposure, A3AR exhibited significant increase in co-localization but was

less evident at 60 min of incubation. Taken together, these results demonstrate that upon internalization, A3AR is transported to the early endosomes and to the lysosomes, suggesting that sequestration occurred mainly within the first 5 min of the exposure to IB-MECA, whereas the distribution to lysosomes occurred later, peaking at 15 min.

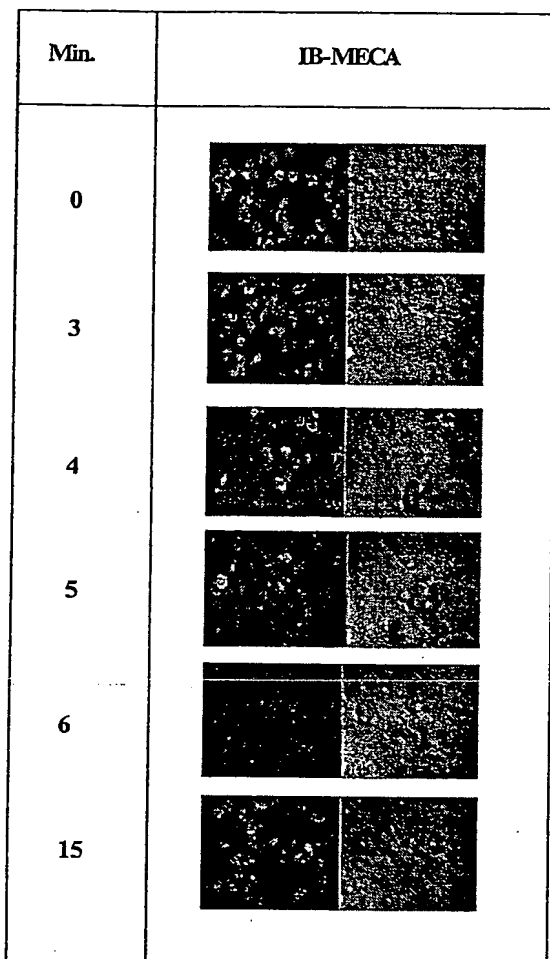
**Radioligand Binding to Surface Receptor of IB-MECA-treated Cells**—To evaluate receptor surface density, IB-MECA-treated cells were exposed to [<sup>125</sup>I]AB-MECA for different time period. Fig. 5 shows that radioligand binding was decreased after 15 (55%) and 60 min (33%), demonstrating that IB-MECA induced accumulated internalization of A3AR. Interestingly, full recovery of the receptor to the cell surface was observed after 24 h.

**RNA and Protein Expression Level of A3AR in IB-MECA-treated Melanoma Cells**—Time-dependent expression of A3AR in the melanoma cells was examined by Western blot analysis. IB-MECA-induced modulation of A3AR expression in a sinusoidal pattern, i.e. down-regulation and up-regulation, occurred at different time points (Fig. 6A). When blocking peptide was utilized, the A3AR band disappeared, confirming that the 32-kDa band is A3AR-specific.

To test whether protein expression was modulated due to degradation and resynthesis, we exposed the cells for 1 h to IB-MECA in the presence of MG132, a protein degradation inhibitor. Indeed, MG132 prevented A3AR down-regulation, illustrating that following internalization, receptor degrada-

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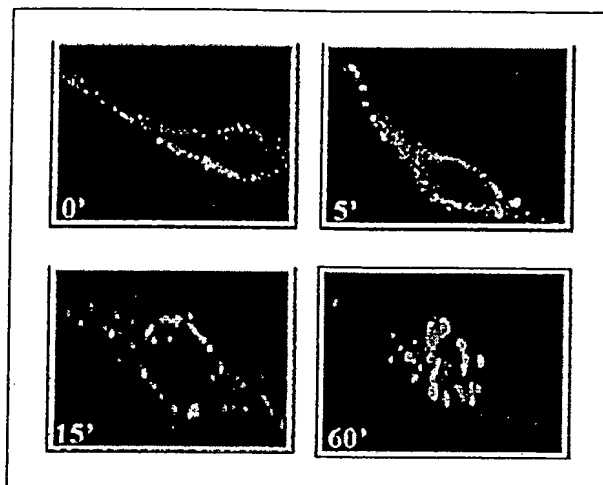
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**FIG. 2. Time course of A3AR internalization/externalization in B16-F10 melanoma cells (confocal microscopy imaging).** B16-F10 melanoma cells were incubated for different time periods at 37 °C with 10 nM IB-MECA. Cells were labeled with the primary antibody against A3AR and the secondary antibody, Cy3-conjugated anti-goat IgG. Images represent the center section of the X-Y plane. Control cells exhibited high fluorescence, gradually disappearing upon IB-MECA treatment for 3, 4, and 6 min. Fluorescence was apparent again after 15 min, when the receptor was externalized to the cell surface.

tion took place (Fig. 6B). We next examined mRNA expression level upon exposure of the cells to IB-MECA for 1 h. Expression level was up-regulated, suggesting that resynthesis of A3AR had occurred (Fig. 6C). The specificity of this response was demonstrated by utilizing the selective antagonist MRS 1523, which reversed the increase in mRNA expression.

**IB-MECA Modulates Key Elements Downstream to A3AR Activation**—To show A3AR functionality in the B16-F10 melanoma cells, we tested cAMP production level and the protein expression level of the downstream effectors PKAc and GSK-3 $\beta$  (known from our former study to be up-regulated upon A3AR activation) (2). IB-MECA inhibited forskolin-stimulated cAMP accumulation after 5 and 15 min, whereas after 30 min, cAMP level was similar to that of the control value (Fig. 7A). Decreased PKAc and increased GSK-3 $\beta$  levels were observed after 15 min, whereas at 30 min, PKAc level stabilized, and GSK-3 $\beta$  only slightly increased (Fig. 7B). The specificity of this response was demonstrated by introducing forskolin to the culture system, which counteracted the effect of IB-MECA and prevented



**FIG. 3. Time course of A3AR internalization/externalization in optical sections of B16-F10 melanoma cells (confocal microscopy imaging).** B16-F10 melanoma cells were incubated for different time periods at 37 °C with 10 nM IB-MECA. Cells were labeled with the primary antibody against A3AR and with the secondary antibody (Cy3-conjugated anti-goat IgG antibody). Images were acquired as single midcellular optical sections at 20 scans/frame. In untreated cells, green fluorescence labeling, representing A3AR, was confined to the cell surface. After 5 min of incubation, green labeling was distributed in the cytosol. At 15 min, the green labeling was less abundant, and at 60 min, green fluorescence was distributed throughout the cytosol and on the cell surface.

the modulation in PKAc and GSK-3 $\beta$  level (Fig. 7C). These results corroborated with the cAMP data, indicating that receptor desensitization/resensitization took place upon chronic exposure to the agonist.

To further evaluate the association between receptor activation, the subsequent downstream signaling events, and the specificity of these responses, B16-F10 melanoma cells were exposed to IB-MECA in the presence and absence of MRS 1523 for 15 min. PKAc and GSK-3 $\beta$  levels were modulated as was described above, leading to down-regulation in the expression level of cyclin D1 and c-myc, the two cell cycle progression genes. MRS 1523 antagonized the modulation in the expression level of the proteins, indicating that the response was mediated via the A3AR (Fig. 8).

**IB-MECA Inhibits Melanoma Development in Mice**—IB-MECA markedly suppressed the development of B16-F10 melanoma tumor growth in the flank model (52% inhibition,  $p < 0.0001$ , Fig. 9A). In mice treated with a combination of IB-MECA and MRS 1523, no inhibition was noted, demonstrating that the antagonist counteracted the activity of IB-MECA and that the response was A3AR-mediated. In tumor lesions excised from these mice, Western blot analysis revealed down-regulation of A3AR, c-Myc, and cyclin D1 and up-regulation of GSK-3 $\beta$  expression level (Fig. 9B). This modulation in the level of proteins was also neutralized by MRS 1523, further demonstrating the specificity of the response.

#### DISCUSSION

IB-MECA is a synthetic A3AR agonist exhibiting a potent antiproliferative effect against tumor cells both *in vitro* and *in vivo* (1, 2, 15, 16). In this study, we show that B16-F10 melanoma cells highly express A3AR. Exposure of the receptor to IB-MECA resulted in receptor internalization/externalization followed by the modulation of key proteins involved in signaling pathways leading to tumor growth inhibition.

Four experimental approaches to test A3AR exhibition and

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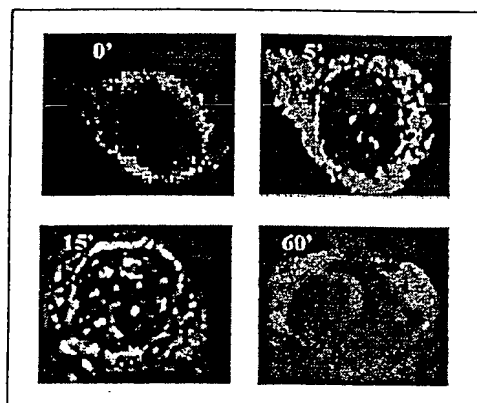
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## Modulation of A3 Adenosine Receptor in Melanoma Cells

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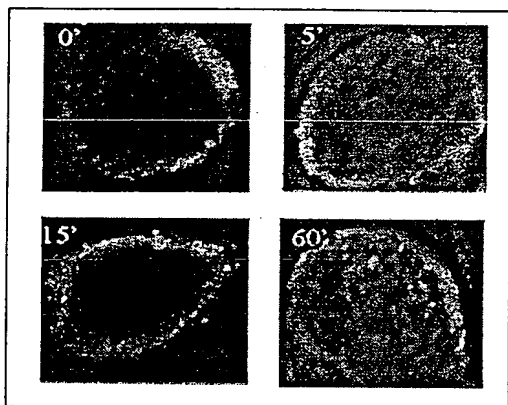


FIG. 4. Time course of A3AR colocalization with transferrin and dextran in B16-F10 melanoma cells exposed to IB-MECA (confocal microscopy imaging). In A, to tag the endosomes, melanoma cells were labeled with 200  $\mu$ g/ml FITC-transferrin (red) in media without serum for 60 min in the presence and absence of IB-MECA (10 nM) for different time periods. The A3AR was labeled as mentioned above (green). In B, lysosomes were labeled by incubating cells with 1 mg/ml FITC-dextran (red) in RPMI with 1% serum at 37 °C for 24 h. Cells were washed and reincubated for an additional 1.5 h in media lacking serum following incubation with IB-MECA (10 nM) for different time periods. The A3AR was labeled as mentioned above (green). For both A and B, stained cells were visualized by a confocal microscope (Zeiss, Axiovert 100 M, excitation at 553 and emission at 568 nm for Cy3, and at 492 and 520 nm, respectively, for fluorescein).

expression in B16-F10 melanoma cells were used in this study. In confocal microscopy analysis, the exhibition of A3AR on the cell surface was exemplified by massive fluorescence, which disappeared on IB-MECA treatment, later to reappear, indicating that receptor internalization/recycling had taken place. The specificity of this response was proved by the introduction of the antagonist MRS 1523 to the culture system in the presence of IB-MECA, resulting in cell surface receptor exhibition similar to the control. The antagonist blocked ligand binding, preventing internalization, thereby retaining full receptor exhibition. Supporting the internalization/externalization event are the studies in which confocal microscopy sectioning exemplified the translocation of the receptor from the membrane to the cytosol. Furthermore, the radioligand binding assay

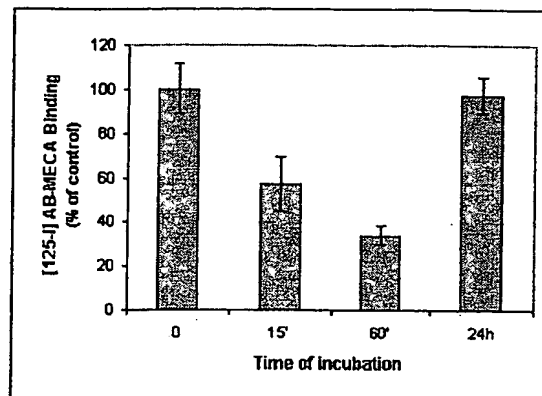


FIG. 5. Cell surface A3AR density in IB-MECA-treated cells as evaluated by [ $^{125}$ I]AB-MECA binding assay. B16-F10 melanoma cells were incubated for different time periods at 37 °C with 10 nM IB-MECA. After incubation, cells were washed to remove agonist. Cell surface A3AR density was evaluated by measuring the radioligand binding at 4 °C for 120 min. Data, expressed as percent of control, represent values of three different experiments.

showed accumulated decrease in surface receptor density upon IB-MECA treatment and full recovery of the receptor to the cell surface after 24 h. Tagging the cells with transferrin confirmed the assumption that the receptor was internalized. Transferrin primarily internalizes with transferrin receptors and constitutively recycles with the receptors through early endosomes to a recycling compartment and then back to the cell surface (10, 17). Our data showing colocalization of A3AR with transferrin after 5 and 15 min, both in the cytoplasm and on the cell surface, support the notion that internalization followed by recycling took place. Taken together, it seems that based on the radioligand binding and the confocal microscopy data, a partial receptor recycling occurs after short incubation period, whereas full recovery takes place after a long period of time. This conclusion may suggest that part of the internalized receptor is subjected to degradation and that a subsequent receptor resynthesis is needed for full receptor recovery. Indeed, the high level of receptor expression was down-regulated shortly after IB-MECA treatment. Prolonged incubation periods resulted in repeated down-regulation/up-regulation of receptor expression, suggesting that this pattern may be a result of receptor degradation and resynthesis. To confirm this notion, we utilized MG132 that prevented receptor down-regulation due to its protease inhibitory effect. Additional data to support the view that part of the internalized receptor was degraded came from confocal microscopy studies in which the cells were labeled with FITC-dextran, which has been shown to specifically accumulate in lysosomes (18). Moreover, the increased expression level of protein and mRNA after 60 min of incubation indicated the involvement of both transcriptional and post-transcriptional events in the process of receptor resynthesis. Others also demonstrated A3AR internalization/recycling; however, the time course did not overlap our values, most probably due to the utilization of different cell types and agonist concentration (19–21).

Receptor functionality was tested by monitoring the level of cAMP and key proteins modulated upon A3AR activation. A decrease in PKAc and an increase in GSK-3 $\beta$  levels were observed both *in vitro* and *in vivo*. The modulation in the level of these proteins was antagonized by forskolin and MRS 1523. Interestingly, after a longer incubation period (30 min), receptor desensitization occurred and was manifested by reversing levels of PKAc and GSK-3 $\beta$ . In a previous study (2), we showed

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## Modulation of A3 Adenosine Receptor in Melanoma Cells

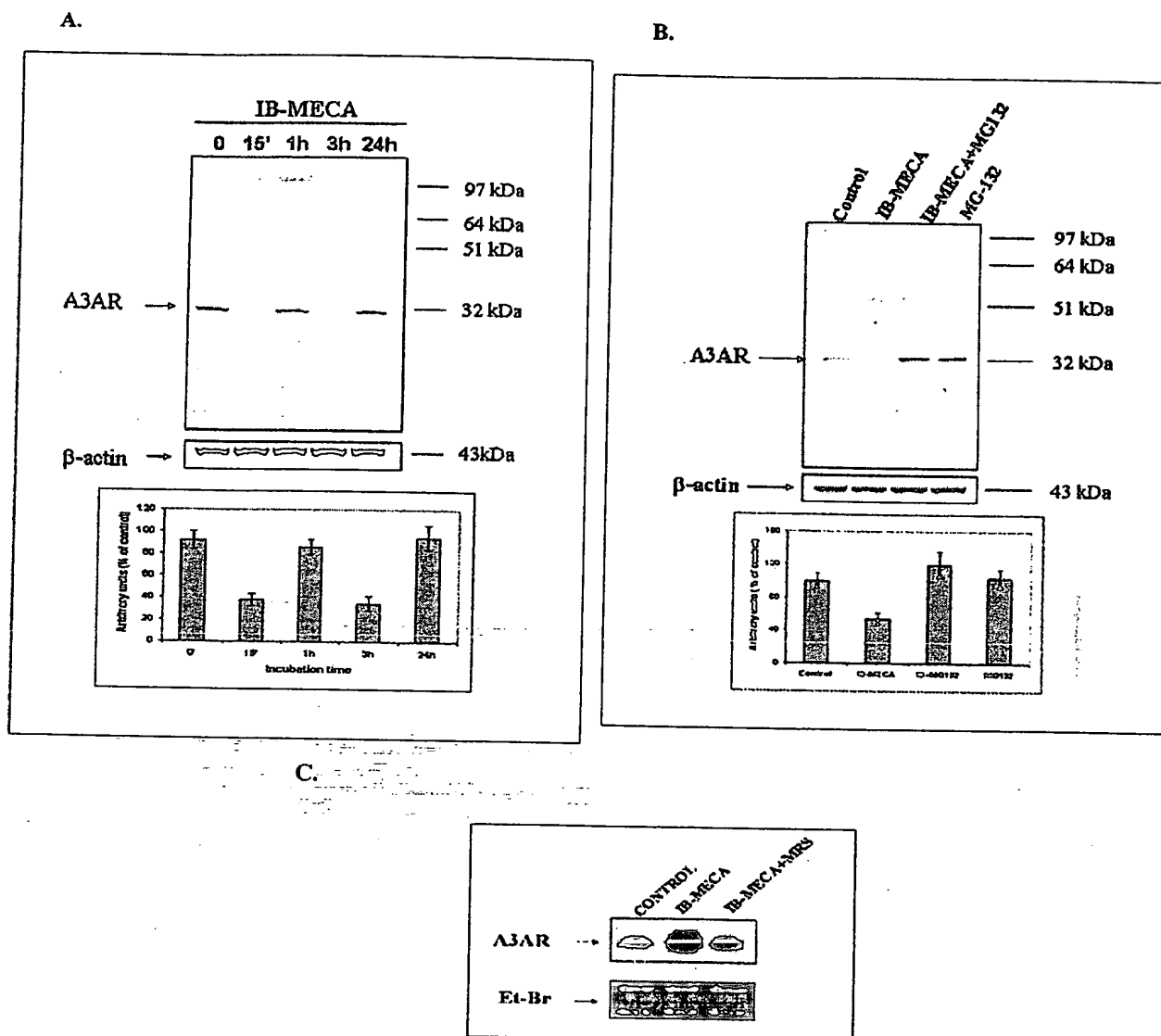


FIG. 6. Protein and mRNA expression level of A3AR upon exposure to chronic IB-MECA treatment. B16-F10 melanoma cells were incubated for different time periods at 37 °C with 10 nM IB-MECA. For A and B, blots were probed with antibodies against A3AR. In A, A3AR expression is down/up-regulated during chronic activation by 10 nM IB-MECA. In B, after 15 min of incubation with IB-MECA, the proteasome inhibitor MG132 prevented A3AR degradation (lane 3). C, Northern blot analysis of A3AR mRNA extracted from control (lane 1), cells treated with IB-MECA for 1 h (lane 2), cells treated with IB-MECA + MRS 1523 for 1 h (lane 3).

that IB-MECA inhibited melanoma cell growth via cross-talk between A3AR and the Wnt signaling pathway. A3AR activation was found to inhibit PKAc and PKB, thereby retaining GSK-3 $\beta$  in its active nonphosphorylated form (2). GSK-3 $\beta$  was shown to phosphorylate and inactivate  $\beta$ -catenin, which consequently induced the down-regulation of c-Myc and cyclin D1 (22). In some tumor cells, including melanoma, GSK-3 $\beta$  fails to phosphorylate  $\beta$ -catenin, which accumulates in the cytosol. It then translocates to the nucleus, where it induces the transcription of cyclin D1 and c-myc, leading to cell cycle progression (7–9). It thus seems that signal transduction pathways initiated upon receptor sensitization also need to be turned off (desensitized) to ensure that signaling can be achieved, allowing the regulation of cell function. Receptor desensitization led to signal termination despite the continuous presence of the agonist in the culture system. Subsequent resensitization, i.e.

the expression of a functional receptor on the cell surface being capable of generating signaling pathways, took place. This chain of events is typical in other G-protein-coupled receptors (23, 24).

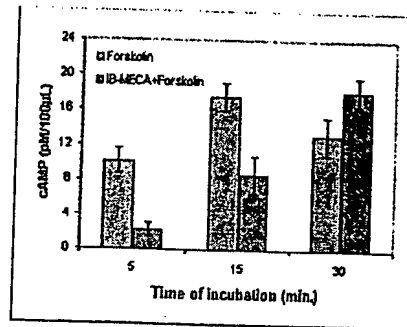
Remarkably, IB-MECA was also efficacious in suppressing melanoma development in mice. The expression profile of A3AR and GSK-3 $\beta$ , in the tumor lesions derived from IB-MECA-treated mice, was similar to that shown *in vitro*. Moreover, cyclin D1 and c-myc levels were down-regulated in the melanoma lesions. These two cell cycle progression genes have been reported earlier to be overexpressed in melanoma cells (25, 26). This suggests their down-regulation as part of the mechanism of melanoma growth inhibition by IB-MECA.

The specificity of tumor suppressive response to IB-MECA was confirmed *in vivo* when melanoma-bearing mice were treated with IB-MECA + MRS 1523. The latter blocked most of

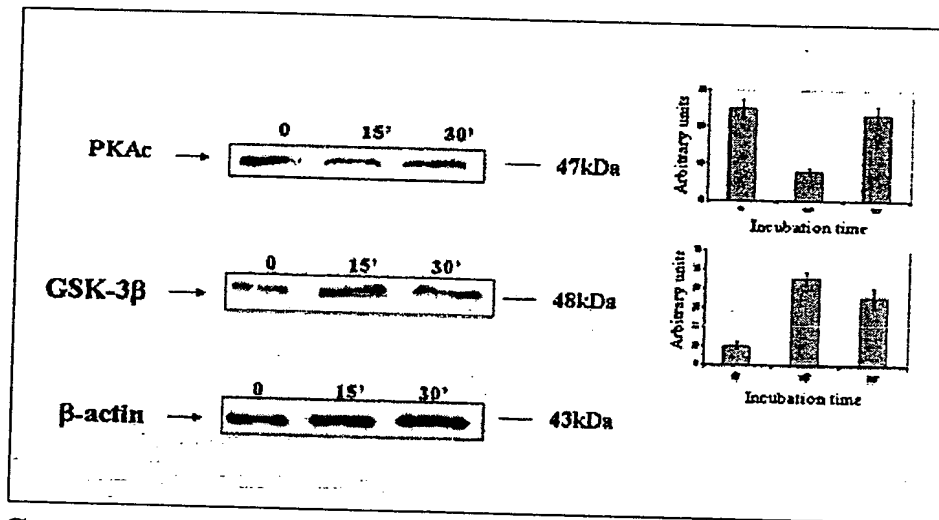
## Modulation of A3 Adenosine Receptor in Melanoma Cells

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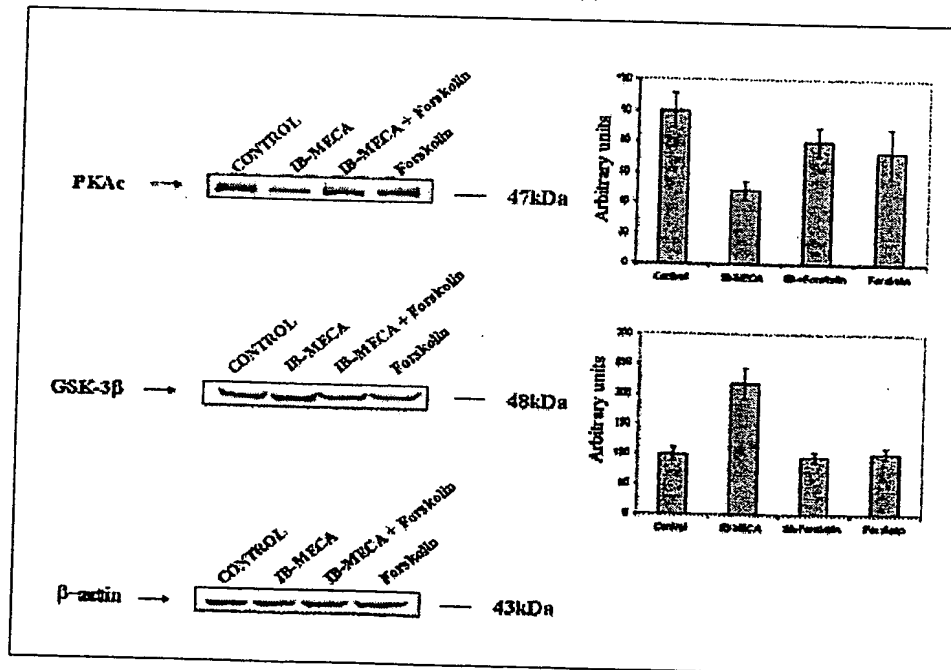
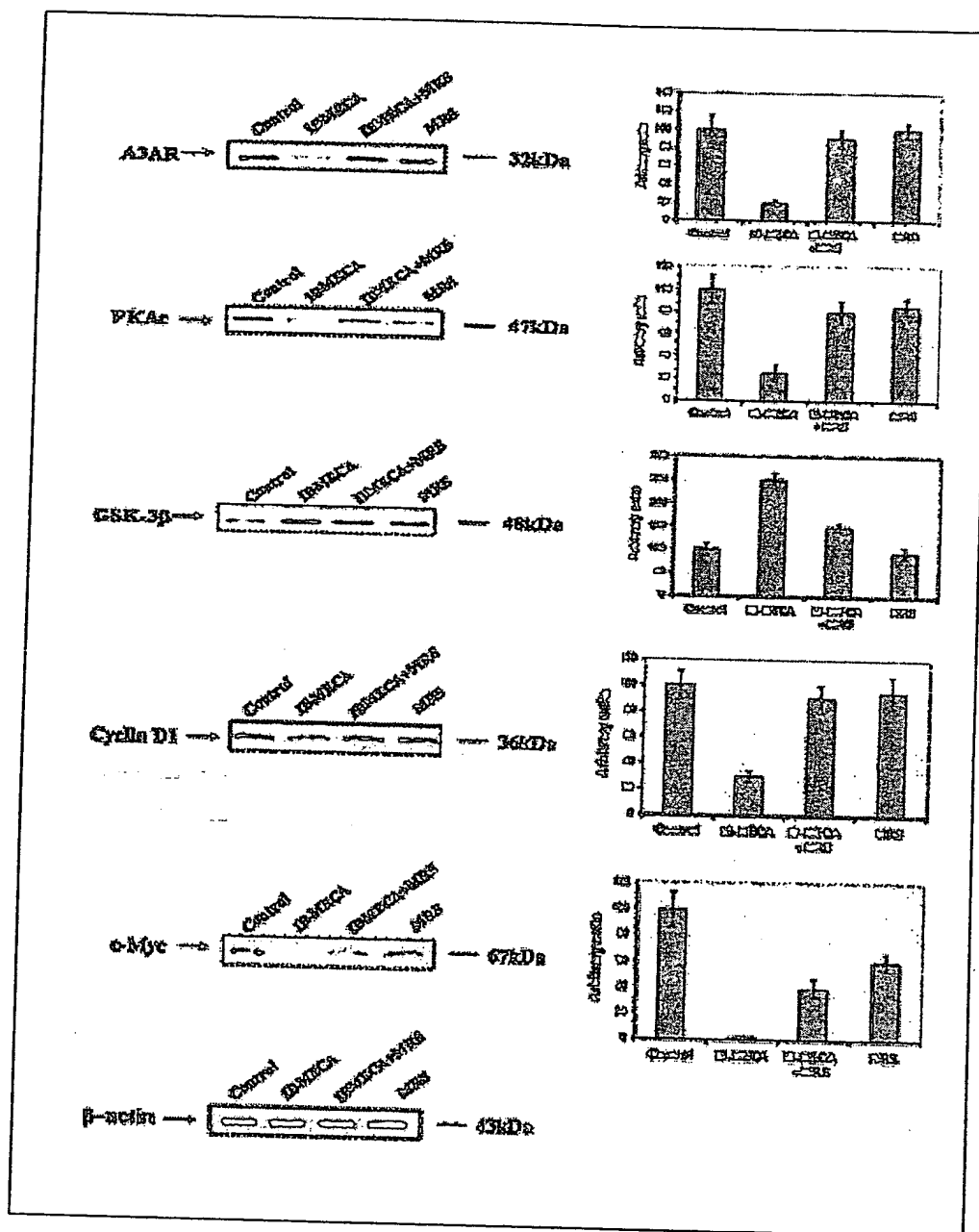


FIG. 7. Receptor functionality is demonstrated by monitoring the level of key elements downstream to A3AR activation. A, effect of IB-MECA (10 nM) on forskolin-stimulated cAMP production. B, immunoblots showing the effect of 10 nM IB-MECA on the expression level of PKAc and GSK-3β in B16-F10 melanoma cells at different time points. C, cells treated (15 min) simultaneously with IB-MECA + forskolin.

## Modulation of A3 Adenosine Receptor in Melanoma Cells



AQ: AA FIG. 8. Modulation of cell growth regulatory proteins in B16-F10 melanoma cells upon IB-MECA treatment. This figure displays immunoblots showing the effect of 10 nM IB-MECA on the expression level of A3AR, PKAc, GSK-3 $\beta$ , c-Myc, and cyclin D1 in B16-F10 melanoma cells. Serum-starved cells (for 18 h) were treated for 15 min with IB-MECA in the presence of 1% fetal bovine serum. To test the specificity of these responses, the antagonist MRS 1523 (100 nM) was introduced to the culture system.

the inhibitory effect of IB-MECA, demonstrating that the response is A3AR-mediated. Earlier studies demonstrated that A3AR agonists such as IB-MECA or Cl-IB-MECA, at micromolar concentrations, induced apoptosis in different cell types. In some of the studies, the activity was found to be A3AR-mediated, whereas in others, A3AR antagonists did not counteract the effect, demonstrating that the apoptosis was not A3AR-mediated (27, 28). In distinction from these studies, in the present work, IB-MECA was used at nanomolar concentrations, and its activity was counteracted by the antagonist

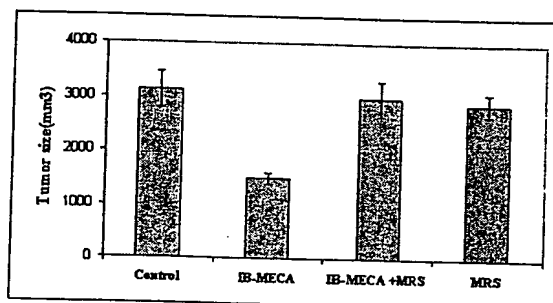
MRS 1523, demonstrating that the response was A3AR-dependent.

In conclusion, melanoma cells highly express and exhibit A3AR. Upon activation, the receptor is internalized to the cytosol, "sorted" to the early endosomes, and recycled to the cell surface. Alternatively, the receptor may be targeted to lysosomes and then subjected to degradation followed by resynthesis and externalization. Modulation of key proteins leading to tumor growth inhibition both *in vitro* and *in vivo* was demonstrated.

## Modulation of A3 Adenosine Receptor in Melanoma Cells

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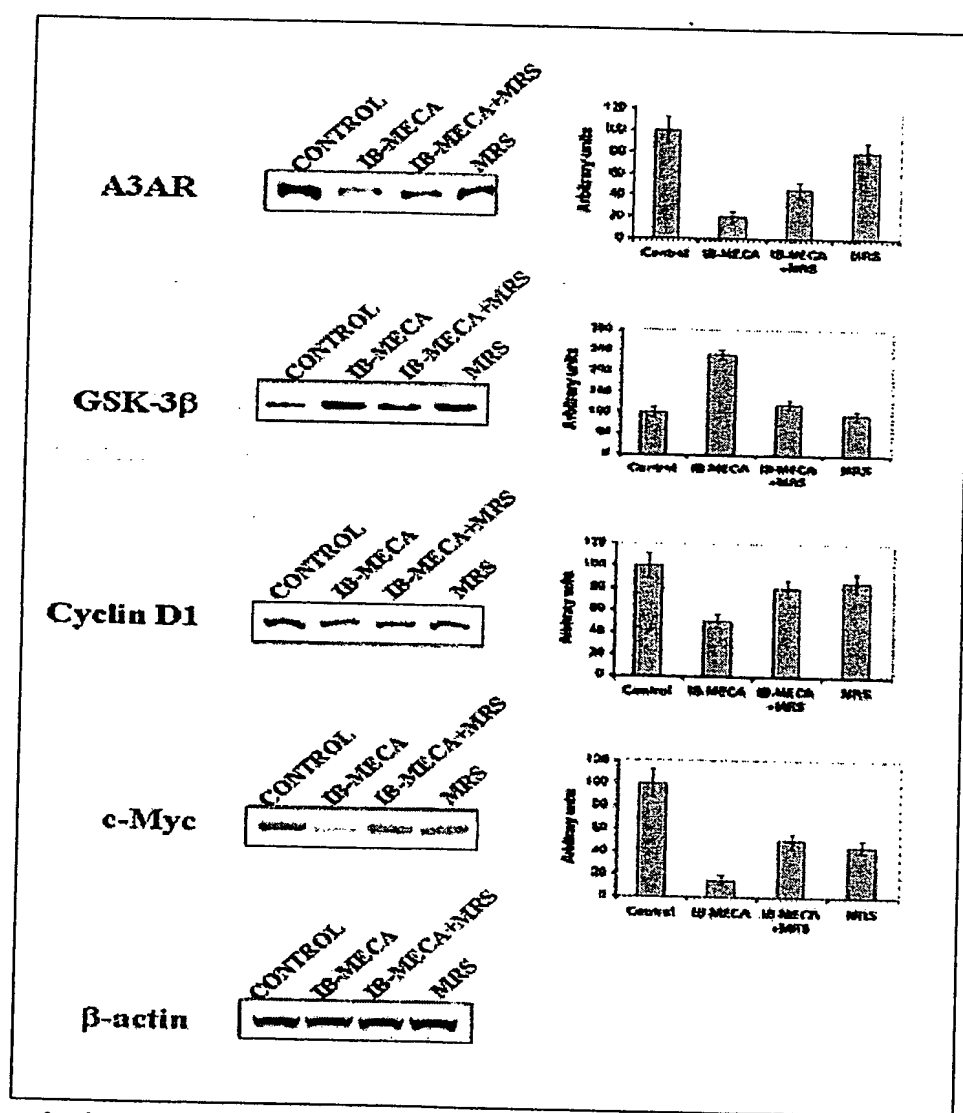


FIG. 9. Inhibition of melanoma cell growth in mice and modulation of cell growth regulatory proteins in tumor lesions. B16-F10 melanoma cells were subcutaneously inoculated to mice and then treated daily with IB-MECA (10  $\mu$ g/kg) or IB-MECA in combination with MRS 1523 (100  $\mu$ g/kg). On day 15, tumor size was measured, and 1 h after IB-MECA treatment, tumor lesions were excised, and then protein was extracted and subjected to Western blot analysis. A, tumor size in the different treated groups (15 mice in each group). IB-MECA inhibited the development of lung metastatic foci ( $p < 0.0001$ ). The antagonist MRS 1523 counteracted the effect of IB-MECA ( $p < 0.005$ ). B, immunoblots showing the effect of IB-MECA and MRS 1523 on A3AR, GSK-3 $\beta$ , cyclin D1, and c-Myc expression level in protein extracts derived from the melanoma tumor lesions.

## Modulation of A3 Adenosine Receptor in Melanoma Cells

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## **ANNEX G**

## ORIGINAL PAPER

An agonist to the A<sub>3</sub> adenosine receptor inhibits colon carcinoma growth in mice via modulation of GSK-3 $\beta$  and NF- $\kappa$ BPnina Fishman<sup>\*1,2</sup>, Sara Bar-Yehuda<sup>1,2</sup>, Gil Ohana<sup>1,2</sup>, Faina Barer<sup>1</sup>, Avivit Ochaion<sup>1</sup>, Abigail Erlanger<sup>1</sup> and Lea Madi<sup>1</sup><sup>1</sup>Can-Fite Biopharma Ltd, Kiryat-Matalon, Petach-Tikva 49170, Israel; <sup>2</sup>Laboratory of Clinical and Tumor Immunology, The Felsenstein Medical Research Center, Tel-Aviv University Sackler Faculty of Medicine, Rabin Medical Center, Petach-Tikva, Israel

A<sub>3</sub> adenosine receptor (A<sub>3</sub>AR) activation with the specific agonist CF101 has been shown to inhibit the development of colon carcinoma growth in syngeneic and xenograft murine models. In the present study, we looked into the effect of CF101 on the molecular mechanisms involved in the inhibition of HCT-116 colon carcinoma in mice. In tumor lesions derived from CF101-treated mice, a decrease in the expression level of protein kinase A (PKA) and an increase in glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) was observed. This gave rise to downregulation of  $\beta$ -catenin and its transcriptional gene products cyclin D1 and c-Myc. Further mechanistic studies *in vitro* revealed that these responses were counteracted by the selective A<sub>3</sub>AR antagonist MRS 1523 and by the GSK-3 $\beta$  inhibitors lithium and SB216763, confirming that the observed effects were A<sub>3</sub>AR and GSK-3 $\beta$  mediated. CF101 downregulated PKB/Akt expression level, resulting in a decrease in the level and DNA-binding capacity of NF- $\kappa$ B, both *in vivo* and *in vitro*. Furthermore, the PKA and PKB/Akt inhibitors H89 and Wortmannin mimicked the effect of CF101, supporting their involvement in mediating the response to the agonist. This is the first demonstration that A<sub>3</sub>AR activation induces colon carcinoma growth inhibition via the modulation of the key proteins GSK-3 $\beta$  and NF- $\kappa$ B.

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Keywords: A<sub>3</sub> adenosine receptor; CF101; colon carcinoma;  $\beta$ -catenin; GSK-3 $\beta$ 

## Introduction

The A<sub>3</sub>AR is a G<sub>i</sub>-protein-coupled receptor containing seven  $\alpha$  helical spanning membrane domains. A<sub>3</sub>AR was found to be expressed in different tumor cell lines, including Jurkat T, pineal gland, astrocytoma, melanoma as well as colon and prostate carcinoma (Gessi *et al.*, 2001; Merighi *et al.*, 2001; Suh *et al.*, 2001; Trincavelli

*et al.*, 2002a; Fishman *et al.*, 2003; Madi *et al.*, 2003; Ohana *et al.*, 2003). A<sub>3</sub>AR activation leads to inhibition of adenylyl cyclase activity, cAMP formation and PKA expression, resulting in the initiation of various signaling pathways which may include the MAPK and the PI3K (Poulsen and Quinn, 1998; Olah and Stiles, 2000; Trincavelli *et al.*, 2002b).

Our earlier studies demonstrated that melanoma cells highly express A<sub>3</sub>AR, and suggested that it may serve as a target for tumor growth inhibition. A<sub>3</sub>AR activation by the synthetic agonist 1-deoxy-1-[6-[(3-iodophenyl)methyl]amino]-9H-purine-9-yl]-N-methyl- $\beta$ -D-ribofuranuronamide (IB-MECA) inhibited the growth of melanoma both *in vitro* and *in vivo* (Fishman *et al.*, 2001, 2002a, b, 2003; Ohana *et al.*, 2001; Madi *et al.*, 2003). The mechanistic pathway involved downregulation of the Wnt signaling pathway. It was found that IB-MECA inhibited the expression of PKAc and PKB/Akt, thereby preventing the phosphorylation and inactivation of GSK-3 $\beta$ . Consequently, GSK-3 $\beta$  was shown to phosphorylate  $\beta$ -catenin and prevent its translocation to the nucleus, resulting in downregulation of cyclin D1 and c-Myc (Fishman *et al.*, 2002b; Madi *et al.*, 2003). PKB/Akt is also known to control NF- $\kappa$ B level by phosphorylating downstream proteins, which in turn release NF- $\kappa$ B from its complex (Madrid *et al.*, 2001). Similar to  $\beta$ -catenin, NF- $\kappa$ B translocates to the nucleus, where, among other genes, it induces the transcription of c-Myc and cyclin D1 (Joyce *et al.*, 2001).

Our previous studies showed that CF101 is efficacious in suppressing the growth of primary and liver metastasis of CT-26 colon carcinoma cells in syngeneic experimental tumor models in mice (Ohana *et al.*, 2003). In addition, CF101 inhibited the growth of subcutaneous HCT-116 human colon carcinoma cells in a xenograft model in mice.

Aberrant activation of Wnt signaling, caused by mutations in  $\beta$ -catenin or APC, is a critical event in the development of colorectal tumors. In these cases, GSK-3 $\beta$  fails to phosphorylate  $\beta$ -catenin, which accumulates in the cytoplasm.  $\beta$ -catenin then translocates to the nucleus where, in association with Lef/Tcf, it induces the transcription of cyclin D1 and c-Myc (Morin, 1999).

The present study is focused on the molecular mechanism involved in the inhibition of colon carcinoma

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ma growth by CF101. We explored the signaling modulation of GSK-3 $\beta$  and NF- $\kappa$ B, both of which are affected by PKB/Akt (which is downstream to PI3K) and are known to regulate the level of the important oncogenes cyclin D1 and c-Myc. A major role for GSK-3 $\beta$  in mediating these responses is discussed.

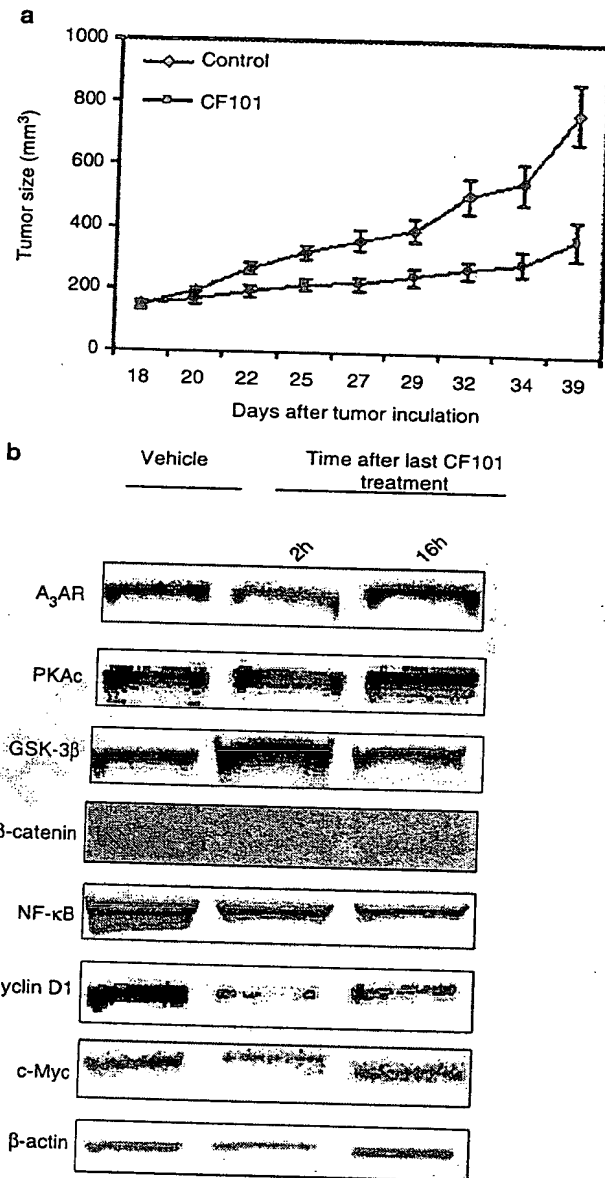
## Results

### *CF101 inhibits colon carcinoma growth in vivo and modulates the expression level of A<sub>3</sub>AR and downstream cell growth-regulatory proteins in tumor lesions*

HCT-116 colon carcinoma cells were engrafted subcutaneously into nude mice. When tumor reached the size of 150–200 mm<sup>3</sup>, the mice were treated daily orally with CF101. Tumor growth was suppressed in the CF101-treated group in comparison to the vehicle-treated group (Figure 1a). On the day of study termination, 52  $\pm$  6.1% ( $P < 0.001$ ) tumor growth inhibition was observed. To evaluate the effect of chronic CF101 treatment on A<sub>3</sub>AR expression and downstream cell growth-regulatory proteins, extracts were prepared from tumor lesions and subjected to Western blot (WB) analysis. In the group of mice killed 2 h after the last treatment, the expression level of A<sub>3</sub>AR, PKAc,  $\beta$ -catenin, NF- $\kappa$ B, c-Myc and cyclin-D1 was downregulated, whereas GSK-3 $\beta$  was upregulated. In the group of mice killed 16 h after the last treatment, A<sub>3</sub>AR expression was similar to that of the vehicle-treated group. Interestingly, in this group, most of the cell growth-regulatory proteins were decreased in comparison to the control group, indicating that continuous downregulation is achieved upon chronic CF101 treatment. Taken together, these data show that receptor downregulation occurs shortly (2 h) after CF101 treatment, leading to modulation of downstream proteins, and that A<sub>3</sub>AR was not desensitized despite chronic activation (over a 20-day period). The expression of the receptor returned to normal levels 16 h after CF101 administration, demonstrating that, even after chronic activation, the receptor is fully expressed (Figure 1b).

### *CF101 modulates the expression level of A<sub>3</sub>AR and downstream cell growth-regulatory proteins in vitro*

To further study the association between A<sub>3</sub>AR activation and the expression of downstream cell growth-regulatory proteins, HCT-116 colon carcinoma cells were incubated in the presence of CF101 (10 nM) for 15 min. Proteins were extracted and analysed by WB. Similar effects of CF101 to those seen *in vivo* were recorded. The expression level of the two kinases PKAc and PKB/Akt was downregulated, while the expression of their downstream substrate GSK-3 $\beta$  was upregulated. The levels of the coactivator  $\beta$ -catenin and the downstream target genes cyclin D1 and c-Myc were decreased (Figure 2a). To confirm that these responses are mediated via the A<sub>3</sub>AR, the antagonist MRS 1523 was introduced to the culture system. The antagonist

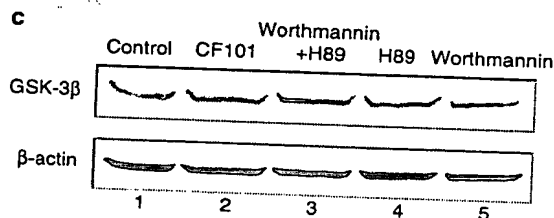
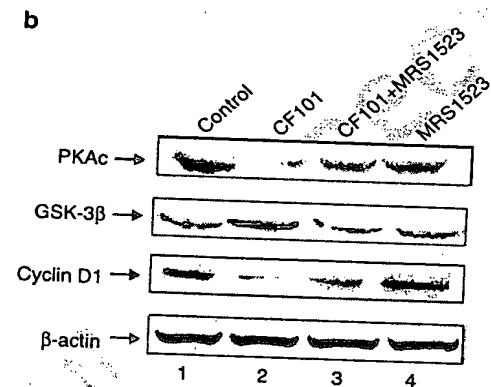
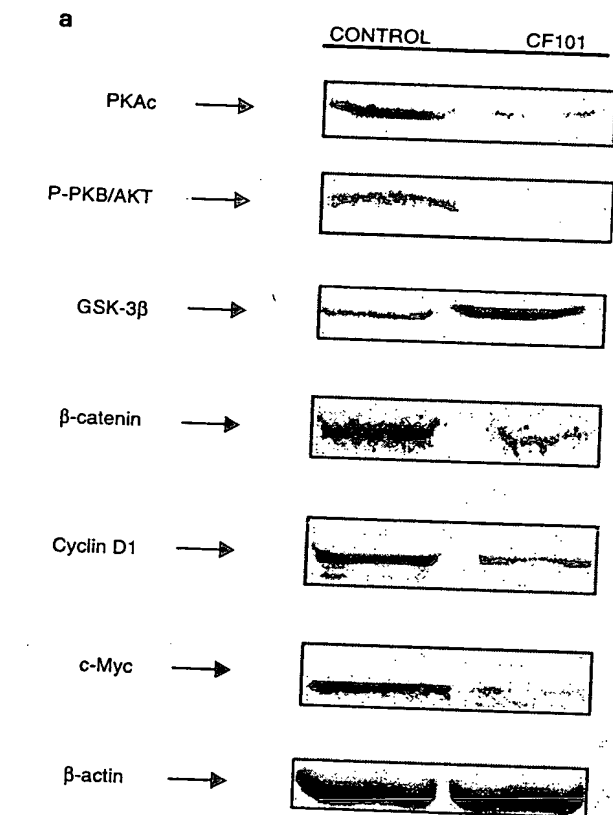


**Figure 1** Inhibition of colon carcinoma cell growth in nude mice and modulation of cell growth-regulatory proteins in tumor lesions. HCT-116 cells were subcutaneously engrafted to nude mice. CF101 (10  $\mu$ g/kg) treatment was initiated when tumor reached a size of 150 mm<sup>3</sup>, and was given twice daily for 21 consecutive days. On day 21, the mice were killed 2 or 16 h after CF101 treatment. Tumor lesions were removed and protein extracts were prepared. (a) Tumor size was measured every 4 days. The curve represents a comparison between the vehicle and CF101-treated groups. (b) Immunoblots showing the effect of CF101 on cell growth-regulatory proteins derived from the colon carcinoma tumor lesions. A<sub>3</sub>AR was downregulated 2 h after treatment and fully expressed after 16 h. Downstream cell growth-regulatory proteins were modulated upon CF101 treatment

counteracted the effect of CF101, thereby retaining the

control levels of PKAc, GSK-3 $\beta$  and cyclin D1, demonstrating the specificity of the response (Figure 2b). To further elucidate the role of PKA and

PKB in mediating cell response to CF101, their activity was mimicked by H89 and Wortmannin (PKA and PKB/Akt inhibitors, respectively). Figure 2c depicts an increase in GSK-3 $\beta$  level upon treatment with the two inhibitors.



#### CF101 deregulates GSK-3 $\beta$ and downstream key signaling proteins

The next set of experiments was carried out to assure that CF101 decreased cyclin D1 and c-Myc levels via modulation of GSK-3 $\beta$ . We therefore compared the active nonphosphorylated GSK-3 $\beta$  level to its nonactive phosphorylated form. Consistent with the former data, we found that, upon CF101 treatment, the nonphosphorylated form was upregulated, whereas the phosphorylated one was decreased (Figure 3a). SB216763, an inhibitor to GSK-3 $\beta$ , counteracted the ability of CF101 to downregulate c-Myc, confirming that this response was GSK-3 $\beta$  mediated (Figure 3b). Furthermore, marked increase in the activity of GSK-3 $\beta$  was also noted after 15 and 30 min (Figure 3c). To assess whether the decrease in  $\beta$ -catenin is mediated via its phosphorylation by GSK-3 $\beta$ , HCT-116 cells were treated with lithium chloride that inhibits the serine/threonine phosphorylation activity of GSK-3 $\beta$ . Indeed, lithium treatment reversed the decrease in  $\beta$ -catenin expression level ( $36 \pm 3.4\%$ ,  $P < 0.002$ ), confirming that this response is GSK-3 $\beta$  mediated (Figure 3d). In addition, the nuclear level of LEF-1 in the CF101-treated cells was downregulated (Figure 3e), supporting the notion that less  $\beta$ -catenin was associated with LEF-1 and subsequently translocated to the nucleus.

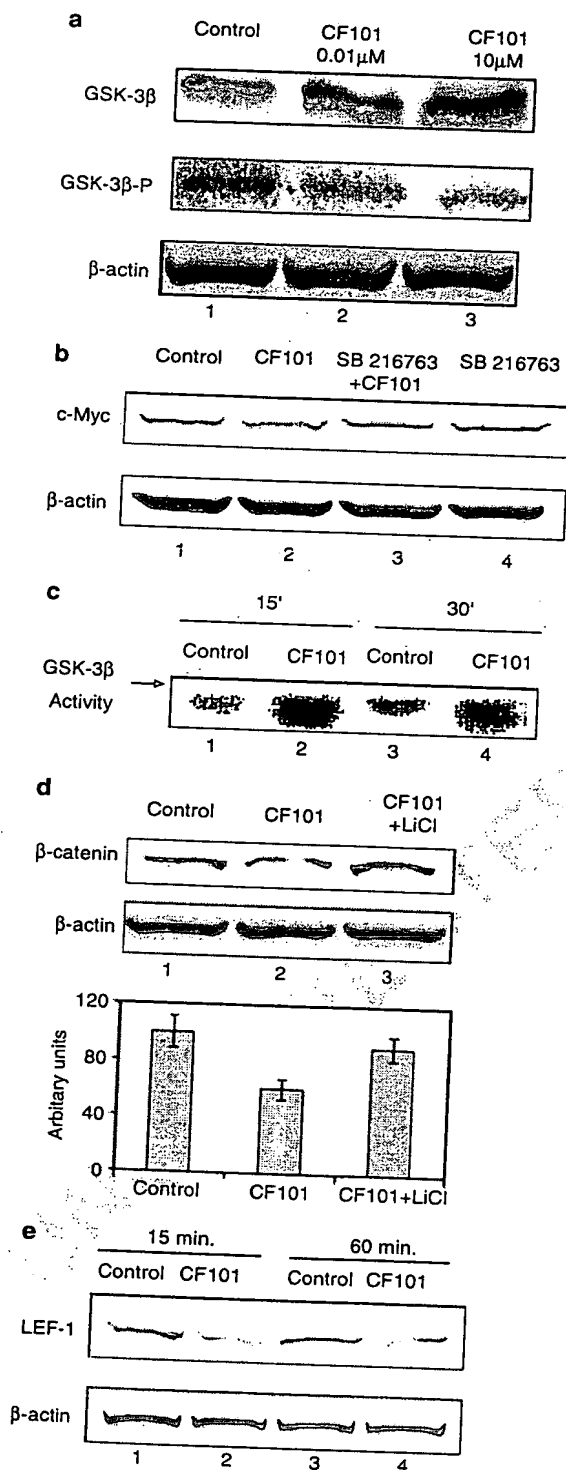
#### Effect of CF101 on the level and transcription activity of NF- $\kappa$ B

Activated PKB/Akt can phosphorylate I $\kappa$ B kinase, leading to further phosphorylation events and the release of NF- $\kappa$ B from its complex with I $\kappa$ B. Accordingly, we examined whether the downregulation of PKB/Akt will affect the protein expression and DNA-binding capacity of NF- $\kappa$ B, also known to induce cyclin D1 and c-Myc transcription. Indeed, decreased NF- $\kappa$ B level was seen in protein extracts derived from CF101-treated HCT-116 cells (Figure 4a). This decrease was blocked when the antagonist MRS 1523 was present in the culture medium together with CF101, demonstrating the specificity of this response. Moreover, electrophore-

**Figure 2** Modulation of cell growth-regulatory proteins in HCT-116 colon carcinoma cells upon CF101 treatment *in vitro*. (a) Immunoblots showing the effect of 10 nM CF101 on the expression levels of PKAc, PKB/Akt, GSK-3 $\beta$ ,  $\beta$ -catenin, cyclin D1 and c-Myc in HCT-116 cells. Serum-starved cells (for 18 h) were treated for 15 min with CF101 in the presence of 1% FBS. (b) To test the specificity of this response, the antagonist MRS 1523 (100 nM) was introduced to the culture system. Immunoblots showing the effect of CF101 on the cell growth-regulatory proteins in the presence and absence of MRS 1523 are depicted. (c) Immunoblots showing the effect of H89 (10  $\mu$ M) and Wortmannin (100 nM) on the expression level of GSK-3 $\beta$

tic mobility shift assay (EMSA) conducted with cell nuclei extracts revealed marked reduction in NF- $\kappa$ B

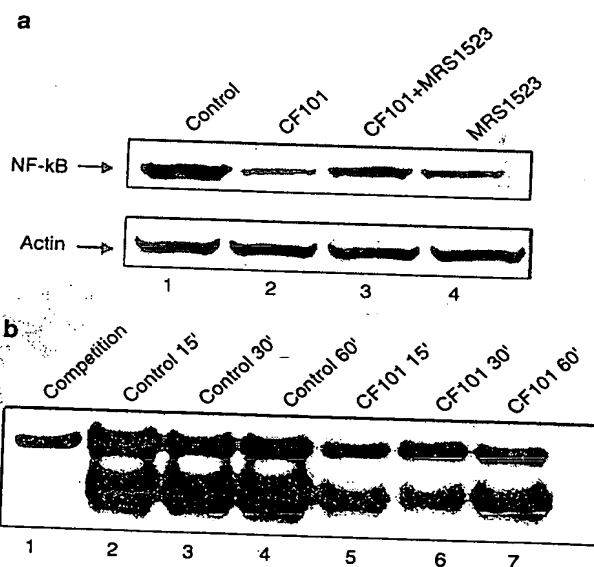
DNA-binding capacity at 15, 30 and 60 min, suggesting a reduction in the NF- $\kappa$ B transcription activity at these time points (Figure 4b).



## Discussion

In the present study, we followed the downstream signaling events taking place subsequent to A<sub>3</sub>AR activation, resulting in tumor growth inhibition. These studies were conducted in a xenograft nude mice model, and were confirmed *in vitro*.

In mice treated chronically for 20 days with CF101, receptor protein downregulation was noted shortly after CF101 administration. Later on, prominent A<sub>3</sub>AR



**Figure 4** NF- $\kappa$ B expression level in cell lysates and EMSA in nuclear extracts. HCT-116 colon carcinoma cells were incubated for 15, 30 and 60 min at 37°C with 10 nM CF101. (a) WB analysis of whole-cell protein extracts conducted at 15 min of incubation in the absence and presence of the antagonist MRS 1523 (100 nM) and (b) EMSA of HCT-116 nuclear extracts at different time points

**Figure 3** Increase in GSK-3β expression level and activity upon treatment of HCT-116 cells with CF101 leads to decreased β-catenin expression level. Cells were depleted from serum for 18 h and treated with vehicle (control) or with CF101 (10 nM or 10 μM) in the presence of 1% FBS for the times and concentrations indicated. (a) The expression of nonphosphorylated GSK-3β and phosphorylated GSK-3β (GSK-3β-P) was determined in cell protein extracts by WB analysis. (b) The ability of CF101 to inhibit the expression level of c-Myc was counteracted by SB216763, an inhibitor of GSK-3β (c). GSK-3β activity in HCT-116 colon carcinoma cells was incubated for 15 and 30 min at 37°C with 10 μM CF101. (d) HCT-116 cells were treated with CF101 (10 nM) for 15 and 30 min in the presence and absence of lithium chloride. The latter counteracted the decrease in β-catenin expression level, indicating that the response is GSK-3β mediated. (e) LEF-1 analysis in the nuclear extracts of HCT-116 cells treated with CF101, as detailed above, for 30 and 60 min

expression was noted, demonstrating that A<sub>3</sub>AR was fully expressed in the tumor cells after chronic treatment with CF101. These fluctuations may be attributed to receptor internalization, degradation and re-synthesis, which occurs subsequent to receptor activation. These data are the first to show A<sub>3</sub>AR expression *in vivo*, and support the notion that colon carcinoma cells do not develop 'resistance' or 'tolerance' to chronic treatment with a synthetic A<sub>3</sub>AR agonist. Supporting the above is our recent publication demonstrating that, upon activation of B16-F10 melanoma cells with IB-MECA, A<sub>3</sub>AR was internalized and sorted to the lysosome for degradation. Later on, the receptor was resynthesized and recycled to the cell surface (Madi *et al.*, 2003).

In the present study, receptor functionality was demonstrated by the modulation in the expression level of key signaling cell growth-regulatory proteins downstream to receptor activation. This included downregulation of PKAc and PKB/Akt and upregulation of GSK-3 $\beta$ . Additionally, the protein expression levels of  $\beta$ -catenin, LEF-1 and the two oncogenes cyclin D1 and c-Myc were found to be decreased.

These results are in accordance with our previous studies, which showed decreased PKAc and PKB/Akt levels upon treatment of B16-F10 melanoma cells with IB-MECA (Fishman *et al.*, 2002b; Madi *et al.*, 2003). PKAc is the catalytic subunit of PKA, known to be activated subsequent to increase in cAMP level. Activation of A<sub>3</sub>AR is known to decrease adenylyl cyclase activity and cAMP formation, resulting in a decline in PKAc level. PKB/Akt has recently been shown to be phosphorylated and thereby activated by PKAc (Fang *et al.*, 2000). The PI3K arm was reported to be upregulated upon A<sub>3</sub>AR activation via the  $\beta\gamma$ -subunit (Schutle and Fredholm, 2002), leading to an increase in the phosphorylated form of PKB/Akt. Here, we show that, in colon carcinoma cells, downregulation of PKB/Akt takes place upon receptor activation, suggesting that in tumor cells modulation of the PKA arm is the dominant event, leading to the downregulation of PKB/Akt. PKAc and PKB/Akt utilize GSK-3 $\beta$  as a substrate and, upon phosphorylation, GSK-3 $\beta$  activity is inhibited. The latter has been widely implicated in cell homeostasis, by its ability to phosphorylate a broad range of substrates including  $\beta$ -catenin, a key component of the Wnt pathway (Ferkey and Kimelman, 2000). In normal cells, GSK-3 $\beta$  phosphorylates  $\beta$ -catenin, thereby inducing its ubiquitination and degradation by the proteasome system (Morin, 1999). However, in tumor cells, GSK-3 $\beta$  fails to phosphorylate  $\beta$ -catenin, leading to its accumulation in the cytoplasm. It then translocates to the nucleus, where it acts in concert with LEF-1 to induce the transcription of the cell cycle progression genes such as cyclin D1 and c-Myc (Kolligs *et al.*, 2002).

In previous studies, we showed that A<sub>3</sub>AR activation induced downregulation of cyclin D1 and c-Myc in melanoma and prostate carcinoma cells, via deregulation of some Wnt signaling proteins (Fishman *et al.*, 2002b, 2003; Madi *et al.*, 2003). We thus assume that the

decreased expression level of  $\beta$ -catenin is responsible for the diminished level of cyclin D1 and c-Myc.

In the present study, we examined the effect of CF101 on HCT-116 colon carcinoma cells, known to be mutated in the  $\beta$ -catenin gene (CTNNB1) (Lovig *et al.*, 2002). Mutations of CTNNB1 were found at the GSK-3 $\beta$  consensus phosphorylation site of  $\beta$ -catenin, that is, a deletion of serine 45 that occurs at a putative phosphorylation target of GSK-3 $\beta$  (Ilyas *et al.*, 1997). Surprisingly, we found that downregulation of  $\beta$ -catenin expression, which occurred upon CF101 treatment, was subsequent to an increase in the level of GSK-3 $\beta$ , notwithstanding the previously described, aforementioned mutation. Moreover, treatment of the cells with lithium, which directly inhibits the activity of GSK-3 $\beta$ , reversed the  $\beta$ -catenin level to that of the control. It thus seems that CF101 circumvents the inability of GSK-3 $\beta$  to phosphorylate  $\beta$ -catenin, leading to its susceptibility to degradation. Support for the involvement of  $\beta$ -catenin in the downregulation of cyclin D1 and c-Myc may be found in the data showing that nuclear level of LEF-1 was downregulated upon CF101 treatment. Furthermore, the GSK-3 $\beta$  inhibitor SB216763 counteracted the ability of CF101 to downregulate c-Myc, thus confirming that the events downstream to  $\beta$ -catenin are also mediated via GSK-3 $\beta$ .

An additional mechanism which may account for the downregulation of c-Myc and cyclin D1 is the direct phosphorylation of the two oncogenes by GSK-3 $\beta$ . It was recently shown that GSK-3 $\beta$  phosphorylates c-Myc at Thr-58 and cyclin D1 at Thr-286, thereby triggering their degradation (Alt *et al.*, 2000; Sears *et al.*, 2000).

The decreased level of PKB/Akt prompted us to examine the involvement of an additional important signaling protein, NF- $\kappa$ B, known to be phosphorylated and activated by PKB/Akt and additional downstream kinases. Since NF- $\kappa$ B is also involved in the transcription of cyclin D1 and c-Myc (Karin *et al.*, 2002), its decreased level may also attribute to the diminished expression of the two cell cycle genes.

The Wnt and the NF- $\kappa$ B signaling pathways are interconnected at the level of cyclin D1 and c-Myc. Both  $\beta$ -catenin and NF- $\kappa$ B control the transcription of these genes, thereby acting as a sensor for growth signals. Taken together, we propose here a model in which activation of the A<sub>3</sub>AR induces modulation of PKAc and PKB, which on one hand upregulates GSK-3 $\beta$ , leading to phosphorylation and ubiquitination of  $\beta$ -catenin. On the other hand, remarkably, the similarity between the *in vitro* and *in vivo* data supports the notion that signaling proteins involved with the Wnt and NF- $\kappa$ B pathways are responsible for the observed modulation of cell growth-regulatory proteins.

The finding that cyclin D1 and c-Myc were downregulated upon A<sub>3</sub>AR activation both *in vitro* and *in vivo* is highly important in light of the bulk literature showing that most human cancers are characterized by overexpression of the two oncogenes (Hosokawa and Arnold, 1998; Parrella *et al.*, 2001; Masuda *et al.*, 2002). In some malignancies, overexpression of these proteins may serve as a marker of poor prognosis (Chana *et al.*,

2002; Nguyen *et al.*, 2003). The importance of these two oncogenes in modulating the tumorigenic response was evidenced by the introduction of an antisense cyclin D1 or c-Myc sequence to malignant cells. This led to the inhibition of growth, the induction of apoptosis and the enhancement of sensitivity to chemotherapeutic agents (Van Waardenburg *et al.*, 1997). Additionally, Jain *et al.* (2002) showed that brief MYC inactivation induced sustained loss of neoplastic phenotype.

Taken together, the molecular model that transpires upon activation of A<sub>3</sub>AR with CF101 includes downregulation of PKAc with a subsequent decrease in PKB/Akt expression level. This may lead on one hand to upregulation of the unphosphorylated form of GSK-3 $\beta$  and the phosphorylation and ubiquitination of  $\beta$ -catenin, resulting in the inhibition of translation of cyclin D1 and c-Myc. Additional events taking place downstream to PKB/Akt include decreased expression and DNA-binding capability of NF- $\kappa$ B, leading also to downregulation of cyclin D1 expression level.

The capability of CF101, a small orally bioavailable molecule, to downregulate cyclin D1 and c-Myc levels both *in vitro* and *in vivo* suggest that the compound is an attractive candidate to be developed as an anticancer agent.

## Materials and methods

### Reagents

CF101 is a GMP grade of the A<sub>3</sub>AR agonist 1-deoxy-1-amino-9H-purine-9-yl]-N-methyl-(D-ribofuranuronamide) (IB-MECA), and was synthesized for Can-Fite BioPharma by Albany Molecular Research Inc., Albany, NY, USA. MRS 1523, a highly selective A<sub>3</sub>AR antagonist, was purchased from RBI/Sigma (Natick, MA, USA). For both reagents, a stock solution of 10 mM was prepared in DMSO and further dilutions in RPMI medium were performed. Lithium chloride and H89 were purchased from Sigma Israel, and SB216763 was purchased from Biomol Research Laboratories Inc. (Plymouth, USA). RPMI, fetal bovine serum (FBS) and antibiotics for cell cultures were obtained from Beit Haemek, Haifa, Israel.

Rabbit polyclonal antibodies against murine and human A<sub>3</sub>AR and the cell growth-regulatory proteins PKAc, PKB/Akt, c-Myc, GSK-3 $\beta$ , phosphor-specific GSK-3 $\beta$  (S9),  $\beta$ -catenin, cyclin D1 and LEF-1 and  $\beta$ -actin were purchased from Santa Cruz Biotechnology Inc., CA, USA.

### Effect of CF101 on the growth of HCT-116 colon carcinoma in nude mice and assessment of A<sub>3</sub>AR expression and cell growth-regulatory proteins in tumor lesions

Mice were maintained on a standardized pelleted diet and supplied with tap water. Experiments were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at Can-Fite BioPharma, Petah Tikva, Israel.

Nude male Balb/c mice, aged 2 months, weighing an average of 20 g, were obtained from Harlan Laboratories, Jerusalem, Israel. HCT-116 colon carcinoma cells ( $1.2 \times 10^6$ ) were subcutaneously injected into the flank of the mice. When tumor reached 150 mm<sup>3</sup> in size, CF101 (10  $\mu$ g/kg body weight) was administered orally twice daily for 20 days. The control

group was treated orally twice daily with the vehicle only. Tumor size (width (*W*) and length (*L*)) was measured twice weekly with a caliber, and calculated according to the following formula: tumor size = (*W*)<sup>2</sup>  $\times$  *L*/2.

After 20 days of treatment and prior to terminating the study, the CF101-treated mice were divided into two groups. (A) mice treated for 20 days with CF101 and killed 16 h after last treatment; (B) mice treated for 20 days with CF101, received additional treatment on day 21 and killed 2 h later. Tumor lesions from the two groups and the control were then excised, homogenized (Polytron, Kinematica) and protein was extracted. WB analysis was carried out to determine the A<sub>3</sub>AR expression level and additional cell growth-regulatory proteins. Each group contained 15 mice and the study was repeated three times. The results depicted are a representative experiment.

### WB analysis

WB analysis of the following samples was carried out: (A) tumor lesions derived from CF101 and vehicle-treated nude mice inoculated with HCT-116 colon carcinoma cells (detailed above). (B) HCT-116 human colon carcinoma cells were serum starved overnight and then incubated with CF101 (10 nM or 10  $\mu$ M) in the presence and absence of MRS 1523 (100 nM), H89 (10  $\mu$ M), Worthmanin (100 nM), and/or SB216763 (1  $\mu$ M) for time periods, as specified below, at 37°C. Samples were rinsed with ice-cold PBS and transferred to ice-cold lysis buffer (TNN buffer, 50 mM Tris buffer pH = 7.5, 150 mM NaCl, 0.5% NP-40). Cell debris were removed by centrifugation for 10 min, at 7500g. Protein concentrations were determined using the Bio-Rad protein assay dye reagent. Equal amounts of the sample (50  $\mu$ g) were separated by SDS-PAGE, using 12% polyacrylamide gels. The resolved proteins were then electro-blotted onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). Membranes were blocked with 1% BSA and incubated with the desired primary antibody (dilution 1:1000) for 24 h at 4°C. Blots were then washed and incubated with a secondary antibody for 1 h at room temperature. Bands were recorded using BCIP/NBT color development kit (Promega, Madison, WI, USA). Data presented in the different figures are representative of at least four different experiments.

### Preparation of nuclear extracts

Nuclear extract proteins from CF101-treated and control HCT-116 cells were prepared by incubating the cells for 15 min on ice in a buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM DTT and 0.5 mM PMSF. Following incubation, Nonident P-40 (10%) was added, cells were vortexed for 10 s and centrifuged. The pellet was resuspended in a buffer containing 20 mM HEPES (pH = 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM DTT and 1 mM PMSF, rocked on a shaker for 15 min at 4°C and centrifuged. Protein was quantified utilizing Bio-Rad protein assay dye reagent.

### GSK-3 $\beta$ immunoprecipitation

HCT-116 human colon carcinoma cells were serum starved overnight and then incubated with CF101 (10  $\mu$ M) for 30 min at 37°C. After isolating protein, 300  $\mu$ g from each sample was removed for immunoprecipitation. The samples were cleared by incubating for 2 h with 1  $\mu$ g/sample of rabbit IgG and 10  $\mu$ l/sample of GammaBind Sepharose (Pharmacia, Piscataway, NJ, USA). After centrifuging, the supernatants were transferred to a tube containing 3  $\mu$ g/sample of Ab against GSK-3 $\beta$  bound to GammaBind Sepharose, and then rotated at 4°C

overnight. The beads were subsequently washed three times with high salt buffer (1 M Tris-HCl pH 7.4, 0.50 M NaCl, and 1% Nonidet P-40) and three times with lysis buffer without protease inhibitors. The immunoprecipitated complexes were used in a kinase activity assay.

#### GSK-3 $\beta$ activity assay

After immunoprecipitating GSK-3 $\beta$  from HCT-116 cells, the protein-containing pellet was washed twice with kinase buffer (20 mM MgCl<sub>2</sub>, 25 mM HEPES, 20 mM glycerophosphate, 20 mM *p*-nitrophenylphosphate, 20 mM sodium orthovanadate and 2 mM DTT). The pellet was then suspended in 20  $\mu$ l kinase buffer and the following ingredients were added: 20  $\mu$ M ATP, 5  $\mu$ Ci ATP (BLU 002Z; DuPont-NEN, Boston, MA, USA) and 10  $\mu$ g myelin basic protein (MBP; Sigma). The total volume of sample plus additions at this point was 25  $\mu$ l. The reaction was continued for 30 min at 25°C and then stopped by the addition of 25  $\mu$ l/sample of 2  $\times$  sample buffer. The samples were boiled for 5 min, then run on a 12% SDS-PAGE gel. The gel was dried, and autoradiography performed to visualize the <sup>32</sup>P-labeled MBP.

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